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(54) Title: ANTITUMOR ANTISENSE SEQUENCES DIRECTED AGAINST R1 AND R2 COMPONENTS OF RIBONUCLEOTIDE REDUCTASE		
(57) Abstract Compounds and methods for modulating cell proliferation, preferably inhibiting the proliferation of tumor cells are described. Compounds that may be used to modulate cell proliferation include inhibitors of ribonucleotide reductase expression, that is, inhibitors of transcription or translation of the gene encoding ribonucleotide reductase. Antisense oligonucleotides complementary to regions of ribonucleotide reductase gene are particularly useful inhibitors.		

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TITLE: ANTITUMOR ANTISENSE SEQUENCES DIRECTED AGAINST R1 AND R2 COMPONENTS OF RIBONUCLEOTIDE REDUCTASE

This application claims benefit under 35 USC §119(e) of United States Provisional Application Serial Number 60/023,040, filed August 2, 1996 and United States Provisional Application Serial Number 60/039,959, filed March 7, 1997.

FIELD OF THE INVENTION

The field of this invention relates to methods of controlling the tumorigenicity and/or metastasis of neoplastic cells. Specifically it relates to the use of antisense sequences directed against the R1 and R2 components of ribonucleotide reductase.

BACKGROUND OF THE INVENTION

The first unique step leading to DNA synthesis is the conversion of ribonucleotides to their corresponding deoxyribonucleotides, a reaction that is catalyzed in a cell cycle specific manner by the housekeeping gene ribonucleotide reductase [Lewis et al., 1978; Reichard, 1993; Wright, 1989a; Wright et al., 1990a; Stubbe, 1989]. The mammalian enzyme is composed of two dissimilar dimeric protein components often called R1 and R2, which are encoded by two different genes located on different chromosomes [Bjorklund et al., 1993; Tonin et al., 1987]. Mammalian protein R1 is a homodimeric structure, with a molecular weight of about 170 kDa, and has substrate sites and allosteric effector sites that control enzyme activity and substrate specificity [Wright, 1989; Thelander et al., 1980; Caras et al., 1985; Wright et al., 1990a]. Protein R2 is a homodimer, with a molecular weight of 88 kDa, and forms two equivalent dinuclear iron centers that stabilizes a tyrosyl free radical required for catalysis [Wright et al., 1990a; Thelander et al., 1985; McClarty et al., 1990]. R1 and R2 proteins interact at their C-terminal ends to form an active holoenzyme [Reichard, 1993; Wright et al., 1990a; Davis et al., 1994].

R1 and R2 are differentially regulated during the cell cycle. There is an S-phase correlated increase in the R2 protein resulting from its *de novo* synthesis [Lewis et al., 1978; Mann et al., 1988]. The activity of ribonucleotide reductase, and therefore DNA synthesis and cell proliferation, is controlled in proliferating cells during the cell cycle by the synthesis and degradation of the R2 component [Eriksson et al., 1984]. The rate-limiting R2 component is a phosphoprotein capable of being phosphorylated by the CDC2 and CDK2 protein kinase mediators of cell cycle progression [Chan et al., 1993], and contains non-heme iron that stabilizes an unique tyrosyl free radical required for enzyme activity [Reichard, 1993; McClarty et al., 1990].

The levels of the R1 protein do not appear to change substantially during the cell cycle of proliferating cells and can be detected throughout the cell cycle. Synthesis of R1 mRNA, like R2 mRNA appears to occur mainly during S phase [Eriksson et al., 1984; Choy et al., 1988; Mann et al., 1988]. The broader distribution of the R1 protein during the cell cycle is attributed to its longer half life as compared to the R2 protein [Choy et al., 1988; Mann et al.,

1988].

Regulation of ribonucleotide reductase, and particularly the R2 component, is altered in malignant cells exposed to tumor promoters or to the growth factor TGF- β [Amara, et al., 1994; Chen et al., 1993; Amara et al., 1995b; Hurta and Wright, 1995; Hurta et al., 1991].

5 Higher levels of enzyme activity have been observed in cultured malignant cells when compared to normal malignant cells [Weber, 1983; Takeda and Weber, 1981; Wright et al., 1989a], and increased levels of R2 protein and R2 mRNA have been found in pre-malignant and malignant tissues as compared to normal control tissue samples [Saeki et al., 1995; Jensen et al., 1994].

10 Regulation of ribonucleotide reductase, and in particular the R2 component, is elevated in transformed cells exposed to tumor promoters, or to transforming growth factor β in growth factor mediated mechanisms of tumor progression [Amara et al., 1996; Chen et al., 1993; Amara et al., 1995b]. These studies are in tumor cells obtained from rodent and human tissues [Weber, 1983; Wright et al., 1989a; Saeki, et al., 1995; Jensen et al., 1994], and in cultured cells
15 selected for resistance to anti-tumor agents such as hydroxyurea [Lewis et al., 1978; Wright et al., 1989b].

Compounds like hydroxyurea inhibit ribonucleotide reductase activity by destabilizing the iron center of the R2 protein causing the destruction of the tyrosyl free radical [McClarty et al., 1990], and preventing cells from progressing through S-phase of the cell cycle [Ashihara and Baserga, 1979].
20

Breakthroughs in molecular biology and the human genome project have opened previously unforeseen possibilities for targeted intervention with mammalian gene expression [Blaese, 1997; Felgner, 1997]. These include approaches such as disruption of specific genes. Antisense (AS) oligonucleotides (AS-ON) designed to hybridize with specific sequences
25 within a targeted mRNA are one example of such targeted intervention. In general, antisense oligonucleotides interact well with phospholipid membranes [Akhter et al., 1991]. Following their interaction with the cellular plasma membrane, they may be actively, or passively, transported into living cells [Loke et al., 1989], and this may occur by a saturable mechanism predicted to involve specific receptors [Yakubov et al., 1989].

30 Many excellent reviews have covered the main aspects of antisense technology and its enormous therapeutic potential. There are reviews on the chemical [Crooke, 1995], cellular [Wagner, 1994] and therapeutic [Hanania, et al., 1995; Scanlon, et al., 1995; Gewirtz, 1993] aspects of this rapidly developing technology. Within a relatively short time, ample information has accumulated about the *in vitro* use of AS-ON in cultured primary cells and cell
35 lines as well as for *in vivo* administration of such ODNs for suppressing specific processes and changing body functions in a transient manner. Further, enough experience is now available *in vitro* and *in vivo* in animal models to predict human efficacy.

It would be useful to have antisense oligonucleotides available to control

tumorigenicity and/or metastatic potential in premalignant or malignant cells wherein the R1 and R2 components of ribonucleotide reductase were utilized.

SUMMARY OF THE INVENTION

The present inventors have shown that aberrant expression of the R2 gene can
5 determine the malignant characteristics of cells. Altered R2 gene expression was found to cooperate with *ras* in mechanisms of malignant progression, and recombinant R2 expression resulted in increased membrane associated Raf-1 protein. These results suggest that R2 cooperates with Raf-1 and Rac-1 thereby affecting *ras* pathways and accordingly cell proliferation and in particular malignant progression.

10 The present inventors also showed that suppression of R2 gene expression reduced transformed properties of tumor cells. In particular, the present inventors demonstrated that novel R2 antisense decreased transformation. R1 antisense also suppressed transformed properties of tumor cells. The R1 and R2 antisense are effective at low concentrations, and surprisingly normal cells were less sensitive to the antisense molecules.

15 Aberrant expression of R2 was also found to result in increased resistance of tumor cells to chemotherapeutic agents. R2 antisense decreased resistance of tumor cells to chemotherapeutic agents at concentrations of antisense that alone did not kill the neoplastic cells.

Broadly stated the present invention relates to compounds and methods for
20 modulating cell proliferation, preferably inhibiting the proliferation of tumor cells. Compounds that may be used to modulate cell proliferation include inhibitors of ribonucleotide reductase expression i.e. inhibitors of transcription or translation of the gene encoding ribonucleotide reductase. Antisense oligonucleotides complimentary to regions of the ribonucleotide reductase gene are particularly useful inhibitors.

25 In one embodiment, the present invention provides an antisense oligonucleotide having a sequence which is complimentary to a nucleic acid sequence from a ribonucleotide reductase gene and comprises at least seven nucleotides or nucleotide analogues. In a preferred embodiment, the oligonucleotide is complimentary to an mRNA region from a ribonucleotide reductase gene, more preferably the ribonucleotide reductase R1 or R2 gene.

30 The invention also relates to a method of evaluating if a compound inhibits transcription or translation of a ribonucleotide reductase gene and thereby effects cell proliferation comprising transfecting a cell with an expression vector comprising a recombinant molecule comprising a nucleic acid sequence encoding ribonucleotide reductase, and the necessary elements for the transcription or translation of the nucleic acid; administering a test
35 compound; and comparing the level of expression of the ribonucleotide reductase with the level obtained with a control in the absence of the test compound.

A method is also contemplated for evaluating a compound for its ability to regulate a Ras signalling pathway by assaying for an agonist or antagonist of the interaction of R2 and

Raf-1 and/or Rac-1 comprising providing a reaction mixture containing R2 and Raf-1 and/or Rac-1 under conditions which permit the interaction of R2 and Raf-1 and/or Rac-1, in the presence of a test compound; detecting the formation of complexes between R2 and Raf-1 and/or Rac-1 or activation of a Ras signalling pathway; and comparing to a control reaction in the absence of the test substance, wherein lower levels of complexes or activation in the reaction mixture indicate that the test compound interferes with the interaction of R2 and Raf-1 and/or Rac-1, and higher levels indicate that the test compound enhances the interaction of R2 and Raf-1 and/or Rac-1.

The present invention also provides a pharmaceutical composition for modulating cell proliferation, preferably tumor cell proliferation, comprising at least one inhibitor of expression of R1 or R2, preferably an antisense oligonucleotide according to the present invention, or a compound identified in accordance with a method of the invention, in admixture with a physiologically acceptable carrier or diluent.

The present invention also contemplates a method of modulating cell proliferation, preferably tumor cell proliferation by contacting a cell with an effective amount of at least one compound that inhibits the expression of R2 or R1, preferably an antisense oligonucleotide according to the present invention, or a compound identified in accordance with a method of the invention.

The present invention also provides a method for reducing cell proliferation, preferably tumor cell proliferation, comprising contacting a cell with an effective amount of an inhibitor of the expression of R1 or R2 preferably, antisense oligonucleotide according to the present invention, or a compound identified in accordance with a method of the invention.

The present invention also provides a pharmaceutical composition for increasing the sensitivity of a tumor cell to a chemotherapeutic drug comprising at least one inhibitor of expression of R1 or R2, preferably an antisense oligonucleotide according to the present invention, or a compound identified in accordance with a method of the invention, in admixture with a physiologically acceptable carrier or diluent. The present invention further provides a pharmaceutical composition for modulating the growth of a tumor cell that is resistant to a chemotherapeutic drug comprising at least one inhibitor of expression of R1 or R2, preferably an antisense oligonucleotide according to the present invention, or a compound identified in accordance with a method of the invention, in admixture with a physiologically acceptable carrier or diluent.

The invention also contemplates the use of an antisense oligonucleotide according to the present invention, or a compound identified in accordance with a method of the invention, to prepare a medicament for modulating cell proliferation.

DESCRIPTION OF THE DRAWINGS

Other advantages of the present invention will be readily appreciated as the same becomes better understood by reference to the following detailed description when considered

in connection with the accompanying drawings wherein:

FIGURE 1A-C are photographs of gels (A and B) and two scans (C) showing the analysis of Myc-tagged R2 expression from stable infectants by Western blot analysis using monoclonal anti-Myc epitope antibody 9E10 (A), polyclonal rabbit anti-R2 serum (B), and during the cell cycle by flow cytometry [Blosmanis et al, 1987; Chadee et al, 1995], using antibody 9E10 (C).

FIGURE 2A-C are photographs (A and B) and a graph (C) of experiments measuring transformed foci wherein (A) shows infection of BALB/c 3T3 (a) and NIH 3T3 (b) cells with SH/mR2 did not lead to focus formation. (B) There was an increase in focus formation with B3/mR2 (b) and N3/mR2 (d) compared to B3/SH (a) and N3/SH (c) after transfection with the T24 H-ras plasmid. (C) The number of foci formed in three independent ras transfection experiments was plotted.

FIGURE 3A-C are photographs of soft agar growth (A) and graphs (B and C) wherein (A) shows expression of Myc-R2 in ras-transformed cells resulted in an increased growth efficiency in soft agar. Examples shown are r-3/mR2 and uninfected r-3 cells (See Table 1). (B) C1/mR2 cells showed reduced tumor latency and increased growth rate when compared to C1/SH control cells where 3×10^5 cells from logarithmically growing cultures were collected and subcutaneously injected into five syngeneic C3H/HeN mice/cell line/experiment. Results presented are from two independent experiments. The p value of t test analysis of tumor growth rates is shown, and indicates that the growth rates for the two cell lines are significantly different. (C) C1/mR2 cells exhibited elevated metastatic potential.

FIGURE 4A-C are graphs wherein (A) shows an increased amount of Raf-1 protein associated with the membrane in R2 overexpressing cells. The recombinant R2 expressing cell lines B3/mR2, N3/mR2, C1/mR2, r-2/mR2, r-3/mR2 and NR4/mR2 (R2) were compared to their respective control lines, B3/SH, N3/SH, C1/SH, r-2/SH, r-3, and NR4 (control). In all cases, cells expressing recombinant R2 exhibited increased membrane associated Raf-1 protein, and when the two groups of cell lines were compared, they were found to be significantly different by t test analysis ($p < 0.001$). (B) Also shows an increase in the activity of mitogen activating protein kinase (MAPK-2) in R2 overexpressing cells. The recombinant R2 expressing lines B3/mR2, N3/mR2, 10T/mR2, C1/mR2, r-2/mR2 and NR4/mR2 (R2) were compared to their respective control lines infected with LXSH (controls). In all cases tested, cells expressing recombinant R2 showed increased enzyme activity, and the difference between two groups was highly significant ($p < 0.001$). (C) Shows increased foci formation with N3/mR2 cells compared to N3/SH cells after transfection with the activated V12 Rac-1 plasmid [Jelinek et al., 1994]. The number of foci shown represents the average \pm SE from two independent experiments.

FIGURE 5A-B are photographs of gels showing examples of Southern blot analysis of CAD (A) and DHFR (B) DNA with mouse L cells. (A) H-4 cells not exposed to drug as a

control (a), H-4 cells from a colony that developed in the presence of 50 μ M PALA (b), or in the presence of 60 μ M PALA (c). DNA was digested to completion with Xba1. (B) SC2 cells not exposed to drug as a control (a), SC2 cells from colonies that developed in the presence of 80 nM methotrexate (MTX) (b) and (c). DNA was digested to completion with Pst1.

5 FIGURE 6A-B are photographs of gels showing examples of Southern blot analysis of CAD (A) and DHFR (B) DNA with BALB/c 3T3 cells. DNA was digested to completion with Pst1. (A) B3/mR2 cells not exposed to PALA (a), and B3/mR2 cells from colonies that developed in the presence of 40 μ M PALA (b), or in the presence of 50 μ M PALA (c). (B) B3/mR2 cells not exposed to MTX (a), and B3/mR2 cells from colonies that developed in the presence of 10 60 nM MTX (b), or in the presence of 80 nM MTX (c).

Figure 7 is a photograph of a Western blot analysis of R2 protein levels in N/R2-4 (a) and N/R2+ASR2 (b) cells. To distinguish the vector R2 protein from the endogenous gene product in transfected cells, a human c-myc epitope coding for ten amino acids plus methionine was placed at the 5' end of the cDNA for R2. Recombinant (upper band) and endogenous (lower 15 band) R2 protein is observed in lane a and is markedly reduced in R2 antisense containing cells (lane b). Both cell lines grew with approximately the same doubling time of about 16 hours.

FIGURE 8 is a photograph of a gel showing p53-DNA binding activity in cells from colonies that developed in the presence of PALA, MTX or hydroxyurea. (a) control 1B cells that are p53-null, (b) B3/mR2 cells that grew in the presence of 20 μ M PALA, (c) B3/R2c2 cells 20 that grew in the presence of 40 μ M PALA, (d) B3/mR2 cells that grew in the presence of 40 nM MTX, (e) B3/R2c2 cells that grew in the presence of 60 nM MTX, (f) B3/mR2 cells that grew in the presence of 0.20 mM hydroxyurea, and (g) B3/R2c2 cells that grew in the presence of 0.30 mM hydroxyurea. Cells were incubated with 32 P-labeled p53 consensus binding sequence in the presence of antibody 421, which activates p53 for DNA binding. Note the presence of 25 complexes in all cell lines except in the 1B control p53-null cells. Low molecular weight complex formation results from p53-DNA binding and high molecular weight complex formation results from antibody supershifted p53-DNA binding.

FIGURE 9 is a graph showing the number of transformation foci in (a) NIH-3T3 mouse cells containing the H-ras oncogene, (b) NIH-3T3 mouse cells containing the H-ras oncogene and 30 the R2 antisense sequence and (c) NIH-3T3 mouse cells containing the H-ras oncogene and the coding region sequence for R2. Results are averages of three experiments.

Figure 10A-B are photographs of a Western blot analysis of AS-II-626-20 inhibition (A) and inhibition by a variety of R2 antisense oligonucleotides (B) of ribonucleotide reductase R2 protein level in L60 mouse tumor cells.

35 DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

1. Antisense and Ribozymes

The present invention provides compounds that inhibit the expression of a ribonucleotide reductase protein and thereby modulate cell proliferation. The compounds may

inhibit the expression of the ribonucleotide reductase by inhibiting the transcription of the gene, or the translation of the mRNA to protein. Such compounds may include antisense oligonucleotides and ribozymes.

The term "antisense oligonucleotide" as used herein means a nucleotide sequence that
5 is complimentary to its target.

The term "oligonucleotide" refers to an oligomer or polymer of nucleotide or nucleoside monomers consisting of naturally occurring bases, sugars, and intersugar (backbone) linkages. The term also includes modified or substituted oligomers comprising non-naturally occurring monomers or portions thereof, which function similarly. Such modified or substituted
10 oligonucleotides may be preferred over naturally occurring forms because of properties such as enhanced cellular uptake, or increased stability in the presence of nucleases. The term also includes chimeric oligonucleotides which contain two or more chemically distinct regions. For example, chimeric oligonucleotides may contain at least one region of modified nucleotides that confer beneficial properties (e.g. increased nuclease resistance, increased uptake into
15 cells), or two or more oligonucleotides of the invention may be joined to form a chimeric oligonucleotide.

The antisense oligonucleotides of the present invention may be ribonucleic or deoxyribonucleic acids and may contain naturally occurring bases including adenine, guanine, cytosine, thymidine and uracil. The oligonucleotides may also contain modified bases such as
20 xanthine, hypoxanthine, 2-aminoadenine, 6-methyl, 2-propyl and other alkyl adenines, 5-halo uracil, 5-halo cytosine, 6-aza uracil, 6-aza cytosine and 6-aza thymine, pseudo uracil, 4-thiouracil, 8-halo adenine, 8-aminoadenine, 8-thiol adenine, 8-thiolalkyl adenines, 8-hydroxyl adenine and other 8-substituted adenines, 8-halo guanines, 8-amino guanine, 8-thiol guanine, 8-thiolalkyl guanines, 8-hydroxyl guanine and other 8-substituted guanines, other
25 aza and deaza uracils, thymidines, cytosines, adenines, or guanines, 5-trifluoromethyl uracil and 5-trifluoro cytosine.

Other antisense oligonucleotides of the invention may contain modified phosphorous, oxygen heteroatoms in the phosphate backbone, short chain alkyl or cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages. For example, the
30 antisense oligonucleotides may contain phosphorothioates, phosphotriesters, methyl phosphonates, and phosphorodithioates. In an embodiment of the invention there are phosphorothioate bonds links between the four to six 3'-terminus bases. In another embodiment phosphorothioate bonds link all the nucleotides.

The antisense oligonucleotides of the invention may also comprise nucleotide analogs
35 that may be better suited as therapeutic or experimental reagents. An example of an oligonucleotide analogue is a peptide nucleic acid (PNA) wherein the deoxyribose (or ribose) phosphate backbone in the DNA (or RNA), is replaced with a polyamide backbone which is similar to that found in peptides (P.E. Nielsen, et al Science 1991, 254, 1497). PNA analogues

have been shown to be resistant to degradation by enzymes and to have extended lives *in vivo* and *in vitro*. PNAs also bind stronger to a complimentary DNA sequence due to the lack of charge repulsion between the PNA strand and the DNA strand. Other oligonucleotides may contain nucleotides containing polymer backbones, cyclic backbones, or acyclic backbones. For example, the nucleotides may have morpholino backbone structures (U.S. Pat. Nol 5,034, 506). Oligonucleotides may also contain groups such as reporter groups, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an antisense oligonucleotide. Antisense oligonucleotides may also have sugar mimetics.

The antisense oligonucleotides may be selected such that they exhibit the least likelihood of dimer formation, self-complementary interactions, and binding potential to the ribonucleotide reductase mRNA other than target sequence. These properties may be determined using the computer modeling program OLIGO Primer Analysis software Version 3.4 (National Biosciences). The program allows the determination of a qualitative estimation of these three parameters and indicates "no potential"; "some potential"; or "essentially complete potential". Oligonucleotides are preferably selected that have estimates of "some potential" or "no potential", most preferably "no potential", in all three parameters as described in Tables 7 and 11. The oligonucleotides are also selected so that their function is not substantially affected by any modifications or substitutions.

The antisense oligonucleotides of the present invention are preferably complimentary to the mRNA region from the ribonucleotide reductase gene. More preferably, the antisense oligonucleotide is complimentary to an mRNA region from the ribonucleotide reductase R2 gene.

The antisense oligonucleotides generally comprise at least seven nucleotides or nucleotides analogs, more preferably, at least 20 nucleotides or nucleotide analogs, most preferably 30-35 nucleotides or nucleotide analogs. The sequences of preferred antisense oligonucleotides according to the present invention can be found in Tables 11 and 7 and are SEQ. ID. NOS. 1-102 and SEQ. ID. NOS. 103-161. More preferred oligonucleotides are shown in Table 12. Most preferred oligonucleotides have the SEQ.ID.NOS 1, 2, 12, 16, 18, 21, 25, 29, 34, 42, 44, 45, 46, 52, 53, 59, 60, 64, 65, 66, 68, 69, 70, 72, 73, 74, 76, 78, 79, 80, 90, 91, 92, 96, 99, 100 and 102 as shown in Table 7.

The antisense oligonucleotides of the invention may be prepared by conventional and well-known techniques. For example, the oligonucleotides may be prepared using solid-phase synthesis and in particular using commercially available equipment such as the equipment available from Applied Biosystems. It is also preferred to substantially purify the oligonucleotides so that they are free of any other factors which would interfere with their activity. Oligonucleotides of the invention may also be identified using genetic complementation techniques, or using the probes described herein. It is also well within the

skill in the art to prepare modified or substituted antisense oligonucleotides.

A ribozyme sequence may also be used to modulate cell proliferation. The ribozyme has homologous or complementary sequences to an antisense oligonucleotide of the invention and the necessary catalytic centre for cleaving the oligonucleotide. The ribozyme type utilized in the present invention may be selected from types known in the art. Several ribozyme structural families have been identified including Group I introns, RNase P, the hepatitis delta virus ribozyme, hammerhead ribozymes, and the hairpin ribozyme originally derived from the negative strand of the tobacco ringspot virus satellite RNA (sTRSV) (Sullivan, 1994, U.S. Patent No. 5,225,347, columns 4 to 5). The latter two families are derived from viroids and virusoids, in which the ribozyme is believed to separate monomers from oligomers created during rolling circle replication (Symons, 1989 and 1992). Hammerhead and hairpin ribozyme motifs are most commonly adapted for trans-cleavage of mRNAs for gene therapy (Sullivan, 1994). Hairpin ribozymes which are presently in clinical trials are preferably used in the present invention. In general the ribozyme is from 30 to 100 nucleotides in length.

2. Methods for Evaluating Compounds

The invention contemplates a method of evaluating if a compound inhibits transcription or translation of a ribonucleotide reductase gene and thereby modulates (i.e. reduces) cell proliferation comprising transfecting a cell with an expression vector comprising a nucleic acid sequence encoding ribonucleotide reductase, the necessary elements for the transcription or translation of the nucleic acid; administering a test compound; and comparing the level of expression of the ribonucleotide reductase with the level obtained with a control in the absence of the test compound.

An expression vector comprising a nucleic acid sequence encoding ribonucleotide reductase may be constructed having regard to the sequence of the gene using procedures known in the art. Suitable transcription and translation elements may be derived from a variety of sources, including bacterial, fungal, viral, mammalian, or insect genes. Selection of appropriate elements is dependent on the host cell chosen, and may be readily accomplished by one of ordinary skill in the art.

Examples of reporter genes are genes encoding a protein such as β -galactosidase (e.g. lacZ), chloramphenicol, acetyl-transferase, firefly luciferase, or an immunoglobulin or portion thereof. Transcription of the reporter gene is monitored by changes in the concentration of the reporter proteins such as β -galactosidase etc. This makes it possible to visualize and assay for expression of recombinant molecules to determine the effect of a substance on expression of the ribonucleotide reductase gene.

Host cells suitable for carrying out the present invention include CHO, COS, BHK, 293 and HeLa. Protocols for the transfection of mammalian cells are well known in the art and include calcium phosphate mediated electroporation, and retroviral, and protoplast fusion-mediated transfection.

The present inventors have found that R2 interacts or cooperates with Raf-1 and/or Rac-1 thereby affecting the Ras signalling pathways. Therefore, the invention also contemplates a method for evaluating a compound for its ability to regulate a Ras signalling pathway by assaying for an agonist or antagonist (i.e. stimulator or inhibitor) of the interaction of R2 and Raf-1 and/or Rac-1. The basic method for evaluating if a compound is an agonist or antagonist of the interaction of R2 and Raf-1 and/or Rac-1, is to prepare a reaction mixture containing R2 and Raf-1 and/or Rac-1 under conditions which permit the interaction of R2 and Raf-1 and/or Rac-1, in the presence of a test compound. The test compound may be initially added to the mixture, or may be added subsequent to the addition of the R2 and Raf-1 and/or Rac-1. Control reaction mixtures without the test compound or with a placebo are also prepared. The formation of complexes or activation of the pathway is detected and the formation of complexes or activation of the pathway in the control reaction but not in the reaction mixture indicates that the test compound interferes with the interaction of R2 and Raf-1 and/or Rac-1. The reactions may be carried out in the liquid phase or R2 and Raf-1 and/or Rac-1, or test compound may be immobilized.

The invention also makes it possible to screen for antagonists that inhibit the effects of an agonist of the interaction of R2 and Raf-1 and/or Rac-1. Thus, the invention may be used to assay for a compound that competes for the same binding site of R2.

The invention also contemplates methods for identifying compounds that bind to proteins that interact with R2 and thereby inhibit R2. Protein-protein interactions may be identified using conventional methods such as co-immunoprecipitation, crosslinking and co-purification through gradients or chromatographic columns. Methods may also be employed that result in the simultaneous identification of genes which encode proteins interacting with R2. These methods include probing expression libraries with labeled R2.

Two-hybrid systems may also be used to detect protein interactions *in vivo*. Generally, plasmids are constructed that encode two hybrid proteins. A first hybrid protein consists of the DNA-binding domain of a transcription activator protein fused to R2, and the second hybrid protein consists of the transcription activator protein's activator domain fused to an unknown protein encoded by a cDNA which has been recombined into the plasmid as part of a cDNA library. The plasmids are transformed into a strain of yeast (e.g. *S. cerevisiae*) that contains a reporter gene (e.g. lacZ, luciferase, alkaline phosphatase, horseradish peroxidase) whose regulatory region contains the transcription activator's binding site. The hybrid proteins alone cannot activate the transcription of the reporter gene. However, interaction of the two hybrid proteins reconstitutes the functional activator protein and results in expression of the reporter gene, which is detected by an assay for the reporter gene product.

It will be appreciated that fusion proteins may be used in the above-described methods. In particular, R2 fused to a glutathione-S-transferase may be used in the methods.

The compounds identified using the method of the invention include but are not

limited to peptides such as soluble peptides including Ig-tailed fusion peptides, members of random peptide libraries and combinatorial chemistry-derived molecular libraries made of D- and/or L-configuration amino acids, phosphopeptides (including members of random or partially degenerate, directed phosphopeptide libraries), antibodies (e.g. polyclonal, 5 monoclonal, humanized, anti-idiotypic, chimeric, single chain antibodies, fragments, (e.g. Fab, F(ab)₂, and Fab expression library fragments, and epitope-binding fragments thereof), and small organic or inorganic molecules. The compound may be an endogenous physiological compound or it may be a natural or synthetic compound.

The reagents suitable for applying the methods of the invention to evaluate 10 compounds that modulate R2 and Raf-1 and/or Rac-1 interactions may be packaged into convenient kits providing the necessary materials packaged into suitable containers. The kits may also include suitable supports useful in performing the methods of the invention.

The compounds identified using the methods described herein, and other inhibitors of R2 expression described herein (e.g. antisense to R2) may be used for modulating a Ras 15 pathway. In particular, the compounds may be used to inhibit the signal-transduction properties of Raf-1 and/or Rac-1; inhibit cell proliferation, alter the cell cycle, and downregulate the immune response in patients with autoimmune diseases. In an embodiment of the invention, the compounds have anti-oncogene or tumor suppressor activity.

3. Methods and Compositions for Modulating Cell Growth/Metastasis

20 The antisense oligonucleotides, ribozymes, and compounds identified using the methods of the invention modulate cell proliferation and in particular tumor cell proliferation. Therefore, methods are provided for interfering with cell proliferation, preferably tumor cell proliferation comprising contacting tissues or cells with one or more of antisense oligonucleotides, ribozymes, and compounds identified using the methods of the 25 invention. Preferably, an antisense oligonucleotide as shown in Table 7 or 11 or 12 is administered.

The term "contact" refers to the addition of an antisense oligonucleotide, ribozyme etc, in a liquid carrier to a cell suspension or tissue sample, or to administering the oligonucleotides etc. directly or indirectly to cells or tissues within an animal.

30 The methods may be used to treat proliferative disorders including various forms of cancer such as leukemias, lymphomas (Hodgkins and non-Hodgkins), sarcomas, melanomas, adenomas, carcinomas of solid tissue, hypoxic tumors, squamous cell carcinomas of the mouth, throat, larynx, and lung, genitourinary cancers such as cervical and bladder cancer, hematopoietic cancers, colon cancer, breast cancer, pancreatic cancer, head and neck cancers, 35 and nervous system cancers, benign lesions such as papillomas, arthrosclerosis, psoriasis, primary and secondary polythemia, mastocytosis, autoimmune diseases, angiogenesis, bacterial infections, and viral infections, such as HIV infections, hepatitis or herpes infections.

The antisense oligonucleotides, ribozymes, and compounds identified using the methods of the invention may also be used to treat drug resistant tumors. Examples of drug resistant tumors are tumors resistant to hydroxyurea; tumors expressing high levels of P-glycoprotein which is known to confer resistance to multiple anticancer drugs such as colchicine, vinblastine and doxorubicin; or, tumors expressing the multi-drug resistance protein as described in R. Deeley et al., Science, 258:1650-1654, 1992.

Antisense oligonucleotides of the invention have also been found to reduce metastasis. In an embodiment of the invention, a method is provided for reducing metastasis in a subject comprising administering an amount of an antisense oligonucleotide of the invention effective to reduce metastasis. Preferably the antisense oligonucleotide has the sequence shown in SEQ. ID. NOS. 1-102 or SEQ. ID. NOS. 103-161, most preferably a sequence shown in Table 12.

Selected antisense oligonucleotides, ribozymes, and compounds may be tested for their ability to modulate cell growth and in particular tumor cell growth, or to reduce metastasis *in vitro* and *in vivo* systems as described herein.

For therapeutic applications, the antisense oligonucleotides, ribozymes, and compounds identified using the methods of the invention may be formulated into pharmaceutical compositions. The pharmaceutical compositions may comprise one or more antisense oligonucleotides, ribozymes, and compounds identified using the methods of the invention for administration to subjects in a biologically compatible form suitable for administration to a subject. The compositions of the invention can be intended for administration to humans and various other mammals, such as ovines, bovines, equines, swine, canines, and felines.

The pharmaceutical compositions of the invention may be administered in different ways depending upon whether local or systemic treatment is desired, and upon the area to be treated. The compositions can be administered orally, subcutaneously or parenterally including intravenous, intraarterial, intramuscular, intraperitoneally, and intranasal administration as well as intrathecal and infusion techniques as required by the malignant cells being treated. For delivery within the CNS intrathecal delivery can be used with for example an Ommaya reservoir or other methods known in the art. The pharmaceutically acceptable carriers, diluents, adjuvants and vehicles as well as implant carriers generally refer to inert, non-toxic solid or liquid fillers, diluents or encapsulating material not reacting with the active ingredients of the invention. Cationic lipids (e.g. Lipofectin, Life Technologies) may also be included in the composition to facilitate oligonucleotide uptake. Implants of the compounds are also useful. In general the pharmaceutical compositions are sterile.

The antisense oligonucleotides and ribozymes of the invention may be delivered using viral or non-viral vectors. Sequences may be incorporated into cassettes or constructs such that an antisense oligonucleotide or ribozyme of the invention is expressed in a cell. Generally the construct contains the proper transcriptional control region to allow the oligonucleotide or

antisense oligonucleotide to be transcribed in the cell.

Therefore, the invention provides vectors comprising a transcription control sequence operatively linked to a sequence which encodes an antisense oligonucleotide or ribozyme of the invention. The present invention further provides host cells, selected from suitable eucaryotic and procaryotic cells, which are transformed with these vectors. Such transformed cells allow the study of the function and the regulation of malignancy and the treatments of the present invention.

Vectors are known or can be constructed by those skilled in the art and should contain all expression elements necessary to achieve the desired transcription of the sequences. Other beneficial characteristics can also be contained within the vectors such as mechanisms for recovery of the nucleic acids in a different form. Phagemids are a specific example of such beneficial vectors because they can be used either as plasmids or as bacteriophage vectors. Examples of other vectors include viruses such as bacteriophages, baculoviruses and retroviruses, DNA viruses, liposomes and other recombination vectors. The vectors can also contain elements for use in either procaryotic or eucaryotic host systems. One of ordinary skill in the art will know which host systems are compatible with a particular vector.

The vectors can be introduced into cells or tissues by any one of a variety of known methods within the art. Such methods can be found generally described in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor Laboratory, New York (1989, 1992), in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, Maryland (1989), Chang et al., *Somatic Gene Therapy*, CRC Press, Ann Arbor, MI (1995), Vega et al., *Gene Targeting*, CRC Press, Ann Arbor, MI (1995), *Vectors: A Survey of Molecular Cloning Vectors and Their Uses*, Butterworths, Boston MA (1988) and Gilboa et al (1986) and include, for example, stable or transient transfection, lipofection, electroporation and infection with recombinant viral vectors.

Introduction of nucleic acids by infection offers several advantages. Higher efficiency can be obtained due to their infectious nature. Moreover, viruses are very specialized and typically infect and propagate in specific cell types. Thus, their natural specificity can be used to target the vectors to specific cell types *in vivo* or within a tissue or mixed culture of cells. Viral vectors can also be modified with specific receptors or ligands to alter target specificity through receptor mediated events.

Additional features can be added to the vector to ensure its safety and/or enhance its therapeutic efficacy. Such features include, for example, markers that can be used to negatively select against cells infected with the recombinant virus. An example of such a negative selection marker is the TK gene that confers sensitivity to the anti-viral gancyclovir. Negative selection is therefore a means by which infection can be controlled because it provides inducible suicide through the addition of antibiotic. Such protection ensures that if, for example, mutations arise that produce altered forms of the viral vector or sequence, cellular

transformation will not occur. Features that limit expression to particular cell types can also be included. Such features include, for example, promoter and regulatory elements that are specific for the desired cell type.

Recombinant viral vectors are another example of vectors useful for *in vivo* introduction of a desired nucleic acid because they offer advantages such as lateral infection and targeting specificity. Lateral infection is inherent in the life cycle of, for example, retrovirus and is the process by which a single infected cell produces many progeny virions that bud off and infect neighboring cells. The result is that a large area becomes rapidly infected, most of which was not initially infected by the original viral particles. This is in contrast to vertical-type of infection in which the infectious agent spreads only through daughter progeny. Viral vectors can also be produced that are unable to spread laterally. This characteristic can be useful if the desired purpose is to introduce a specified gene into only a localized number of targeted cells.

A vector to be used in the methods of the invention may be selected depending on the desired cell type to be targeted. For example, if breast cancer is to be treated, then a vector specific for such epithelial cells should be used. Similarly, if cells of the hematopoietic system are to be treated, then a viral vector that is specific for blood cells and their precursors, preferably for the specific type of hematopoietic cell, should be used.

Retroviral vectors can be constructed to function either as infectious particles or to undergo only a single initial round of infection. In the former case, the genome of the virus is modified so that it maintains all the necessary genes, regulatory sequences and packaging signals to synthesize new viral proteins and RNA. Once these molecules are synthesized, the host cell packages the RNA into new viral particles which are capable of undergoing further rounds of infection. The vector's genome is also engineered to encode and express the desired recombinant gene. In the case of non-infectious viral vectors, the vector genome is usually mutated to destroy the viral packaging signal that is required to encapsulate the RNA into viral particles. Without such a signal, any particles that are formed will not contain a genome and therefore cannot proceed through subsequent rounds of infection. The specific type of vector will depend upon the intended application. The actual vectors are also known and readily available within the art or can be constructed by one skilled in the art using well-known methodology.

If viral vectors are used, for example, the procedure can take advantage of their target specificity and consequently, do not have to be administered locally at the diseased site. However, local administration may provide a quicker and more effective treatment, administration can also be performed by, for example, intravenous or subcutaneous injection into the subject. Injection of the viral vectors into a spinal fluid can also be used as a mode of administration. Following injection, the viral vectors will circulate until they recognize host cells with the appropriate target specificity for infection.

Transfection vehicles such as liposomes can also be used to introduce the non-viral vectors described above into recipient cells within the inoculated area. Such transfection vehicles are known by one skilled within the art.

The pharmaceutical compositions and vectors of the invention may be administered
5 in combination with other drugs or singly, consistent with good medical practice and treatment modalities that are known in the art. Examples of other drugs which may be administered in combination with the compositions etc. of the invention are cytotoxic agents, immunotoxins, alkylating agents, anti-metabolites, antitumor antibiotics and other anti-cancer drugs

Dosing of the antisense oligonucleotides, ribozymes, and compounds will depend on
10 the severity and responsiveness of the condition to be treated with a course of treatment lasting from several days to several months or until diminution of the disease is achieved. Optimal dosing schedules may be calculated using measurements of drug accumulation in the body. Persons of ordinary skill in the art can readily determine optimum dosages, dosing methodologies, and repetition rates. Optimum dosages may vary depending on the relative
15 potency of individual oligonucleotides, and can generally be determined based on ED₅₀s *in vitro* and *in vivo* animal studies. The pharmaceutical compositions or vectors of the invention, and combination drugs may each be administered at non-cytotoxic or cytotoxic doses, or one may be administered at a cytotoxic dose and the other at a non-cytotoxic dose. The doses may be selected to provide a synergistic effect.

20 EXAMPLES

The examples provide an analysis of malignancy related characteristics of cells containing deregulated R2 expression achieved by gene transfer techniques. Overexpression of R2 leads to an increased frequency of transformed foci formation by mouse fibroblasts following transfection with activated H-*ras*. In addition, expression of recombinant R2 in *ras*-
25 transformed cells resulted in enhanced colony forming efficiency in soft agar, and markedly elevated tumorigenic and metastatic potential *in vivo*. Furthermore, deregulated R2 expression can cooperate with other oncogenes like *rac-1* in mechanisms of transformation.

The results herein demonstrate for the first time that the R2 component of mammalian ribonucleotide reductase is a malignancy determinant that can synergize with
30 activated oncogenes to modify malignant potential, and supports a model in which these effects are mediated through alterations in major Ras pathways that are brought about by deregulated R2 gene expression. The observations presented here indicated that R2 can also participate in other critical cellular functions, and can play a direct role in determining malignant potential through oncogene cooperativity.

35 The examples further demonstrate that ribonucleotide reductase R2 gene expression can play a significant role in determining drug sensitivity characteristics, and that this appears to occur at least in part through a mechanism involving genomic instability.

The mechanism through which aberrant R2 expression modifies drug sensitivities

does not appear to require the direct involvement of p53 mutation or loss of wild type p53 function, although it is possible that genetic events downstream of a p53 regulated pathway are involved. As shown in Example 1 a relationship exists between increased R2 expression and activation of a *ras* pathway involving the Raf-1 protein and mitogen-activated protein kinase-2 (MAPK) activity. Recombinant R2 gene expression in Balb/c 3T3 and NIH-3T3 cells significantly increases both Raf-2 protein activation and mitogen-activating protein kinase (MAPK) activity.

A hypothesis for the above observations can be made, but it is not to be construed as limiting the present invention to this one mode of action. These observations imply that the R2 protein is capable of acting as a signal molecule in the MAPK pathway, in addition to its role as a rate-limiting component of ribonucleotide reduction. Transcription factors like the product of the *c-myc* gene are downstream targets of the MAPK pathway, and control for example, expression of cyclins A, D and E, which are important in the regulation of checkpoints during cell cycle progression [Hunter, 1994; 1995]. Compromising cell cycle checkpoint controls enhance genomic destabilization and facilitates DNA amplification [Kohn, 1996; Livingston et al., 1992]. *c-myc* overexpression has also been directly linked to gene amplification mechanisms involving DHFR [Mai, 1994]. These observations suggest that alterations in the MAPK pathway through aberrant R2 expression may be at least partly responsible for the observed changes in drug sensitivities and genomic integrity.

Example 3 demonstrates that short antisense sequences directed against the R1 and R2 components have anti-tumor activity and are cytotoxic to the neoplastic cells. Further, the R2 antisense sequences can also act synergistically with well known chemotherapeutic agents. Very low concentrations (non-toxic) of short antisense sequences reduced the resistance of the neoplastic cells to chemotherapeutic agents such as N-(phosphonacetyl)-L-aspartate (PALA) and methotrexate (MTX) as well as hydroxyurea. As shown in the Example, cells were transfected with a vector containing the R2 sequence in an antisense orientation. These cells were more sensitive to the chemotherapeutic agents. Also, mouse 10T¹/₂ cells which are drug resistant, when transfected with R2 sequence in the antisense orientation, were found to have significantly reduced resistance (increased sensitivity) to the chemotherapeutic agents. Short synthetic antisense sequence complementary to the R2 sequence also provided increases sensitivity.

The above discussion provides a factual basis for the use of antisense oligonucleotides and ribozymes directed against the R2 mRNA. The methods used with and the utility of the present invention can be shown by the following non-limiting examples and accompanying figures.

GENERAL METHODS:

GENERAL METHODS IN MOLECULAR BIOLOGY: Standard molecular biology techniques known in the art and not specifically described were generally followed as in Sambrook et al.,

- Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor Laboratory, New York (1989, 1992); in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, Maryland (1989); and in Perbal, *A Practical Guide to Molecular Cloning*, John Wiley & Sons, New York (1988). Polymerase chain reaction (PCR) was carried out generally as
- 5 in *PCR Protocols: A Guide To Methods And Applications*, Academic Press, San Diego, CA (1990).

Vectors can be constructed for the present invention by those skilled in the art and should contain all expression elements necessary to achieve the desired transcription of the sequences. The expression elements can be selected to allow expression only in the cell being

10 targeted. Other beneficial characteristics can also be contained within the vectors such as mechanisms for recovery of the nucleic acids in a different form. One of ordinary skill in the art will know which expression elements are compatible with a particular cell type. The vectors can be introduced into cells or tissues by any one of a variety of known methods within the art as described herein above.

- 15 GENERAL METHODS IN IMMUNOLOGY: Standard methods in immunology known in the art and not specifically described were generally followed as in Stites et al.(eds), *Basic and Clinical Immunology* (8th Edition), Appleton & Lange, Norwalk, CT (1994) and Mishell and Shiigi (eds), *Selected Methods in Cellular Immunology*, W.H. Freeman and Co., New York (1980).
- 20 ASSAYS FOR TUMORIGENICITY AND METASTASIS: Malignancy potential was determined as reported previously [Wright, 1989a; Egan et al., 1987a, 1987b; Damen et al., 1989; Taylor et al., 1992; Stokoe et al., 1994]. Six to eight week old C3H/HeN syngeneic mice (Charles River, Quebec) were used to evaluate tumorigenic and metastatic potential of the cells. Cells were prepared from subconfluent, logarithmically growing cultures, collected by
- 25 gentle treatment with trypsin/EDTA solution and adjusted to appropriate concentration in a balanced salt solution.

For the tumorigenicity (tumor latency) assay, 1×10^5 cells in a 0.1 ml volume were injected subcutaneously into the back of mice and the time required to form a tumor (2×2 mm) detectable by palpation was recorded. The growth of tumors was also evaluated by measuring

30 tumor diameters, and estimating tumor base area each day following tumor appearance [Damen et al., 1989]. Tumor size was determined by multiplying the dimensions of the cross-section of the tumor. Tumors were removed from the mice and tumor weight was recorded 21 days later. In the case of no tumor formation, mice were kept for 2 months after injection and then sacrificed.

- 35 For experimental metastasis assays (determination of metastatic potential), 1×10^5 cells in a 0.2 ml volume were injected into the tail veins of 6-8 week old C3H/HeN syngeneic mice and an estimate of the number of lung tumors was made 21 days later. The mice were sacrificed, and the lungs were stained by injecting Bouin's solution (picric acid, formaldehyde,

acetic acid (15:5:1) intratracheally [Egan et al., 1987b; Damen et al., 1989]. Pulmonary tumors were counted with the aid of a dissecting microscope. To confirm that equal numbers of test and control cells were injected, duplicate culture plates containing growth medium were inoculated with 100 cells per plate. After 10 days in culture, plates were stained with methylene blue and colonies were scored.

RIBONUCLEOTIDE REDUCTASE ASSAY: Ribonucleotide reductase activity in crude extracts prepared from cells is assayed as previously described [Lewis et al., 1978; Hurta and Wright, 1992; Hurta et al., 1995]. Enzyme preparations are obtained from logarithmically growing cells lysed in phosphate buffered saline, pH 7.2, containing 1 mM dithiothreitol and 1 mM protease inhibitor, AEBF (Calbiochem, San Francisco, CA), by three cycles of freeze-thawing. Following centrifugation, the supernatant is used for enzyme activity assays with [¹⁴C]-CDP (Moravsek Biomedical, Brea, CA), as detailed previously [Lewis et al., 1978; Hurta and Wright, 1992; Fan et al., 1996; Choy et al., 1988].

WESTERN BLOT ANALYSIS: The procedures used have been reported [Fan et al., 1996a; 1996b; Choy et al., 1988]. Briefly, following cell extract preparation, total protein content was determined, and an aliquot was analyzed on 10% linear SDS-polyacrylamide gel. After protein transfer and blocking, membranes were incubated with anti-R2 rabbit polyclonal antibody. Alkaline phosphatase conjugated goat anti-rabbit IgG (Sigma) was used for protein R2 detection.

EXAMPLE 1

R2 COOPERATES WITH ACTIVATED ONCOGENES

To determine the malignant potential of deregulated expression of the rate-limiting R2 component of ribonucleotide reductase, the properties of cells stably infected with a retroviral expression vector (SH/mR2) carrying the R2 component [Fan et al., 1996b], were investigated. Further the interaction between R2 and activated oncogenes was explored.

MATERIALS AND METHODS

Expression Vectors: The retroviral expression vector for the human Myc epitope-tagged mouse R2 component, SH/mR2, was constructed and packaged as described in Fan et al [1996b]. The infectivity of the viral stock was $>1 \times 10^4$ colony-forming units/ml. Plasmid pH06Ti which expresses T-24 H-ras and a selective marker *neo* was used for malignant transformation [Egan et al., 1987a, 1987b; Taylor et al., 1992]. The activated Rac-1 plasmid (V12 Rac-1) was kindly provided by M. Symons [Stokoe, et al., 1994].

Cells and Cell Culture: The mouse cell lines, BALB/c 3T3, NIH 3T3, four lines of T24 H-ras transformed $10T^{1/2}$ cells, named C1, NR4, r-2 and r-3 have been previously used as recipients of the R2 retroviral vector [Fan et al., 1996b]. Cells were routinely cultured in α -minimal essential medium (α -MEM)(Gibco, Grand Island, NY) supplemented with 10% calf serum (Fetalclone III, Hyclone, Logan, UT). Infection of cells with SH/mR2 or control virus LXSH in the presence of polybrene was carried out [Miller et al., 1993], and stable infectants ($>1 \times 10^4$

clones) were obtained with hygromycin selection and pooled [Fan et al., 1996b; Miller et al, 1993]. Determinations of cell division times, plating efficiencies, and relative sensitivities to hydroxyurea cytotoxicity by estimating relative colony forming efficiencies, were carried out as previously described [Lewis et al., 1978; Egan et al., 1987a; Hards and Wright, 1981].

- 5 Growth in soft agar was estimated in 10 cm tissue culture plates containing 15 ml base agar (0.5% Bacto-agar in α -MEM plus 10% calf serum) and 10 ml of growth agar (0.33% agar in α -MEM containing 10% calf serum). Cells were obtained from subconfluent cultures, and colonies were scored 10-15 days later [Egan et al., 1987a, 1987b; Hards and Wright, 1981]. Transformation was also analyzed by determining focus formation after cells were infected with SH/mR2 or LXSH or transfected with T-24 Ras or V12 Rac-1 plasmids by calcium phosphate precipitation [Taylor et al, 1992]. At 40 hours after infection or transfection, cells were split into three 10 cm tissue culture plates which were provided daily with 20 ml of fresh complete medium (α -MEM plus 10% calf serum) for 10-14 days, stained with methylene blue and foci were scored [Taylor et al, 1992]. The transfection frequency in all the experiments were routinely determined by cotransfection of a mammalian expression plasmid for β -galactosidase from *Escherichia coli*, with the T-24 Ras or V-12 Rac-1 plasmids, followed by treatment of cells with the X-gal and counting the number of blue cells [Price et al, 1987]. In some cases, T-24 Ras plasmid transfected plates were selected with geneticin, and drug resistant colonies were scored approximately 14 later after staining with methylene blue.

- 20 **Assays for Tumorigenicity and Metastasis:** Malignant potential was determined as described herein above.

- Protein R2 Analysis:** The procedures for Western blot analysis have been described previously, for example, using either the anti-myc mouse monoclonal 9E10 antibody (ATCC, Rockville, MD) [Fan et al., 1996b] or the anti-R2 rabbit polyclonal antibody [Chan et al., 1993]. To determine recombinant R2 protein expression during the cell cycle, flow cytometry analysis was performed following 9E10/fluorescein isothiocyanate antibody labelling as previously described [Blosmanis et al, 1987; Chadee et al, 1995].

- Determination of Membrane-associated Raf-1 Protein:** The membrane fraction was prepared as described by Qui et al. [1995], and used for Western analysis with a polyclonal antibody specific for Raf-1 protein (Santa Cruz Biotechnology Inc., Santa Cruz, CA), after the protein content was determined by the standard Bio-Rad assay. Densitometry analysis of the Raf-1 band was performed, and the amount of Raf-1 protein from each sample was corrected by densitometry analysis of a well separated band on a parallel gel stained with Coomassie blue.

- Ribonucleotide Reductase Assay:** The Assay was performed as described herein above. In some experiments enzyme assays were performed by combining purified recombinant R1 protein [Salem et al, 1993] with 9E10 antibody-precipitated R2 protein [Hurta and Wright, 1992]. In this Example, 20 μ g of the 9E10 antibody and 50 μ l of Staphylococcal protein A-agarose (Sigma Chem. Co., St. Louis, MO) were added to 1 ml of the supernatant of centrifuged lysed

cells, and placed on a rocker at 4°C for 2 hours. The Staphylococcal protein A agarose-immunocomplex was washed three times with 1 ml of cold phosphate buffer containing 1 mg/ml bovine serum albumin. The immunocomplex was then assayed for ribonucleotide reductase activity [Lewis et al, 1978; Hurta and Wright, 1992; Fan et al., 1996b; Choy et al, 1988].

Assay of MAPK Activity: Cultures with >90% confluency were stressed in serum-free medium [Stokoe et al., 1994; Jelinek et al, 1994] and extracted as previously described [Alessi et al., 1995]. MAPK-2 protein was immunoprecipitated by agarose beads conjugated with non-neutralizing antibody for the protein (Santa Cruz Biotechnology, Inc.), and the kinase activity of the immunocomplex was assayed by measuring its ability to phosphorylate myelin basic protein using a MAPK assay kit from Upstate Biotechnology, Inc. (Lake Placid, NY).

RESULTS

Expression of Biologically Active R2 Protein. To determine the malignant potential of deregulated expression of the rate-limiting R2 component of ribonucleotide reductase, the properties of cells stably infected with a retroviral expression vector (SH/mR2) carrying the R2 component [Fan et al., 1996b], were investigated. The use of this expression vector allowed high infection efficiency and stable expression of the R2 protein. To distinguish the vector gene product from the endogenous R2, a human c-Myc epitope coding for 10 amino acids plus methionine was added to the 5'-end of the R2 cDNA. Figure 1A shows that Western blots with the 9E10 antibody that specifically recognizes the Myc-epitope sequence detects the R2 protein of approximately 45 kDa in SH/mR2 stably infected BALB/c 3T3 and NIH 3T3 cells (named B3/mR2 and N3/mR2, respectively), but not in control vector (LXSH) infected B3/SH or N3/SH cells. R2 specific antibodies detected the endogenous as well as the recombinant R2 protein in expression vector infected cells, and as expected only the endogenous protein was observed in control vector infected cells (Fig. 1B).

Flow cytometry analysis following 9E10/fluorescein isothiocyanate antibody labelling demonstrated that the recombinant R2 protein was constitutively expressed throughout the cell cycle (Fig. 1C). Indirect microscopic analysis using the 9E10 antibody indicated that essentially every cell in the B3/mR2 and N3/mR2 populations expressed the Myc-tagged R2 protein.

Several experiments were performed to demonstrate that the vector-expressed R2 is biologically active. First, B3/mR2 and N3/mR2 cells were observed to be resistant in colony-forming experiments to the cytotoxic effects of hydroxyurea, an inhibitor of the R2 protein [Wright, 1989; Wright et al., 1989], when compared to B3/SH and N3/SH cells [Fan et al., 1996b]. Second, ribonucleotide reductase activity was assayed and found that the CDP reductase activities in B3/mR2 and N3/mR2 cells in three independent experiments were 1.96 ± 0.32 and 1.71 ± 0.11 nmoles/mg protein/hour, respectively, which was 2.6 and 2.1 times higher than observed with B3/SH and N3/SH cells (0.74 ± 0.14 and 0.83 ± 0.08 nmoles/mg/hour,

respectively). Finally, enzyme assays were carried out by combining purified recombinant R1 protein [Salem et al, 1993], with 9E10 antibody precipitated R2 protein. Significant levels of activity (15 to 20 nmoles/mg/hr.) were detected when B3/mR2 and N3/mR2 cells were used as a source for Myc-tagged R2, and as expected no activity was found when B3/SH or N3/SH cells were used.

Ras Transformation Potential Determined by Aberrant R2 Gene Expression. The above results indicate that cells can be altered in the regulation of biologically active R2 protein. Therefore, altered R2 expression was tested to see if it further transformed cells like BALB/c 3T3 or NIH 3T3. Similar to control B3/SH and N3/SH cells, as well as the parental non-infected lines, B3/mR2 and N3/mR2 cultures remained in a flat, non-transformed morphology on tissue culture plates, and exhibited contact and density inhibited growth (data not shown). No transformed foci were observed with BALB/c 3T3 or NIH 3T3 cells after infection with the retroviral SH/mR2 vector (Fig. 2A, a and b).

The results suggest that deregulation of R2 gene expression does not on its own transform BALB/c 3T3 or NIH 3T3 fibroblasts. To test the hypothesis that deregulated R2 expression may cooperate with oncogenes line *H-ras*, an expression plasmid containing T24 *H-ras* was transfected into established recombinant R2 expressing cell populations derived from BALB/c 3T3 or NIH 3T3. A consistent and significant increase (3.4 fold) in the number of foci formed with *H-ras* transfected N3/mR2 cells was observed when compared to N3/SH control cells (Fig. 2B, c and d and Fig. 2C). An even more marked increase of about 70 fold was observed when *H-ras* transfected B3/mR2 cells were compared to B3/SH cells (Fig. 4B, a and b and Fig. 2C). This occurred even though the transfection efficiency with N3/mR2 and B3/mR2 cells as determined by scoring G418 selected colonies, and/or counting blue cells following cotransfection of *H-ras* with an expression plasmid for *E. coli* β -galactosidase [Price et al., 1987], were actually lower (by about 50%) than with N3/SH and B3/SH cells.

Ras Malignancy Potential Determined by Aberrant R2 Gene Expression. Since combinations of altered R2 gene expression and activated *H-ras* were synergistic in focus forming experiments in which *ras* was transfected into altered R2 expressing cells, this gene combination was tested further by infecting four independent *H-ras* transformed 10T¹/₂ cell lines, C1, NR4, r-2 and r-3 that were previously characterized [Egan et al., 1987a, 1987b; Taylor et al., 1992; Stokoe et al., 1994], with the retroviral vector SH/mR2. Stable infectants were selected with hygromycin, and Western blot analyses and enzyme activity assays confirmed that these infectants expressed biologically active Myc-tagged R2 protein.

Soft agar growth experiments revealed that *H-ras* transformed cells containing the recombinant R2 sequence were much more efficient at producing colonies in semi-solid growth agar than the uninfected parental populations (e.g. r-3) or control vector infected cells (C1, NR4, r-2) (Table 1). In addition, many of the colonies formed by cells infected with recombinant R2 were larger in size (Fig. 3A). Since each pair of recombinant R2 expressing and

control cell populations have almost identical growth rates (12.9 hours for C1/SH and 12.2 hours for C1/mR2, 13.5 hours for r-2/SH and 13.9 hours for r-2/mR2, 11.6 hours for r-3 and 11.9 hours for r-3/mR2, 14.1 hours for NR4/SH and 14.3 hours for NR4/mR2), plating efficiencies (58% for C1/SH and 55% for C1/mR2, 59% for r-2/SH and 63% for r-2/mR2, 91% for r-3 and 88% for r-3/mR2, 73% for NR4/SH and 75% for NR4/mR2), and cell cycle phase distributions (data not shown) when grown on solid surfaces, the alterations observed in soft agar and in foci forming experiments suggest that a combination of deregulated R2 expression and activated H-ras may lead to greater malignant potential *in vivo*.

Therefore, the tumorigenic and metastatic potential of C1/mR2 and C1/SH cells was compared in syngeneic C3H/HeN mice. Marked differences in malignant potential were observed. C1/mR2 cells exhibited shorter tumor latency and greater tumor growth when compared to C1/SH cells (Fig. 3B). Furthermore, metastasis assays clearly indicated that C1/mR2 cells were more malignant than C1/SH cells and produced significantly more lung tumors (Fig. 3C).

R2 Gene Expression and Oncogene Cooperativity. The above results indicate that altered R2 expression can cooperate with activated H-ras in *in vitro* transformation and in *in vivo* malignancy assays. Since no obvious differences in growth rates or cell cycle phase distributions were found that may account for this cooperation, as for example changes in cell cycle regulation, the following idea was tested. Does deregulated R2 expression synergize with *ras* by elevating the activity of a Ras signal pathway? This would be consistent with studies showing a direct correlation between *ras* expression and malignant potential [Egan et al., 1987a, 1987b; Wright et al, 1993; Bradley et al, 1986]. A major Ras pathway for regulating gene expression involves the Raf-1 protein kinase. Activated Ras recruits Raf to the plasma membrane where Raf and downstream signalling molecules like MAPKs become activated [Stokoe et al, 1994; Jelinek et al, 1994; Leever et al, 1994].

Using a Raf-1 specific antibody, the levels of membrane associated Raf-1 in six BALB/c 3T3, NIH 3T3 and 10T 1/2 derived cell lines containing deregulated R2 expression was compared with control cells containing only endogenous R2 protein (Fig. 4A). In all six cases, cell lines containing deregulated R2 showed increased membrane associated Raf-1, with an average increase of about 30% which was highly significant ($p < 0.001$). In agreement with the above observation, cell lines with deregulated R2 expression exhibited a consistent and significant increase of about 70% ($p < 0.001$) in MAPK-2 activity (Fig. 4B). Oncogenic Ras also activates the Rac pathway which is parallel to the Raf pathway, and therefore constitutively active Rac-1 cooperates with membrane-targeted Raf-1 in malignant transformation [Qiu et al, 1995].

If MAPK activation mediated by Raf-1 translocation and activation is important in the R2/*ras* synergism described herein above in this Example, then aberrant R2 expression should cooperate with activated Rac-1 in cellular transformation, because it has been shown

- previously that activated Raf-1 and Rac-1 cooperate in mechanisms of transformation [Qiu et al, 1995]. Figure 4C shows that this prediction is correct, since positive cooperation in transformation between activated Rac-1 and R2 was observed in a manner similar to Ras and R2, as measured by focus formation with N3/mR2 and N3/SH cells transfected with activated V12 Rac-1 [Qiu et al, 1995]. These observations are consistent with the view that deregulated R2 gene expression cooperates with oncogenes like *ras* and *rac* by upregulating Raf translocation and MAPK pathway activity, but they do not rule out the possibility that other transduction pathways involving activated Raf may also be involved, since there is evidence that Raf can regulate some cellular activities through MAPK-independent pathway(s) [Lenormand et al, 1996; Koong et al, 1994; Agarwal et al, 1995].

- This Example indicates for the first time that the R2 component of mammalian ribonucleotide reductase is a novel malignancy determinant that can synergize with activated oncogenes to modify malignant potential. It is important to note that the only role ascribed to R2 in the cell prior to this Example is as a rate-limiting component of ribonucleotide reductase. This Example demonstrates that R2 can also participate in other critical cellular functions and can play a direct role in determining malignant potential through oncogenic cooperativity.

EXAMPLE 2

R2 GENE EXPRESSION AND CHANGES IN DRUG SENSITIVITY AND GENOME STABILITY

20 MATERIALS AND METHODS

- Cell Lines and Culture Conditions:* The hydroxyurea resistant mouse cell lines, H-2, H-4, LHF and SC2 were derived from mouse L cells and have been characterized in Choy et al [1988] and McClarty et al [1986]. BALB/c 3T3 cells were used as recipients of an R2 retroviral expression vector (B3/mR2 and B3/R2c2 cell lines), or of the same retroviral vector lacking the R2 sequence (B3/SH cells) [Fan et al., 1996a; 1996b]. NIH-3T3 cells were also used as recipients of the R2 retroviral expression vector (N/R2-4 cell line) or of this retroviral vector lacking the R2 sequence (N/SH cells), as described previously [Fan et al., 1996a; 1996b]. The N/R2+ASR2 cell line was the recipient through co-transfection using LipofectAmine (Life Technologies, N.Y) [Damen et al., 1991] of retroviral vectors containing the R2 coding sequence and the R2 sequence in the antisense orientation. RP3 and RP6 cells are 10T¹/2 mouse cells that have been transfected with the T-24 H-*ras* oncogene and a mutant oncogenic form of the p53 gene [Taylor et al., 1992], and they were also used as recipients through transfection using LipofectAmine reagent, of a retroviral vector containing the R2 coding region in an antisense orientation [Fan et al., 1996b], to obtain RP3/ASR2 and RP6/ASR2 cells. 1B cells are p53^{-/-} and were derived from embryonic fibroblasts [Lowe et al., 1994]. All cells were cultured in α -minimal essential medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum (Intergen, Purchase, NY) and antibiotics (100 units/ml penicillin and 100 μ g/ml streptomycin) at 37°C in a humidified atmosphere containing 5% CO₂.

Drug Selections: Cells ranging in numbers from 500 to 1.2×10^5 were added to 100 mm tissue culture plates in growth medium containing 10% dialyzed fetal bovine serum, and in the absence or presence of drug [Huang et al., 1995a; Choy et al., 1988]. The culture medium was replaced with fresh medium every week for two to three weeks. Surviving cells were visualized by methylene blue staining, and colonies of about 50 cells or more were scored [Huang et al., 1995a]. The relative colony forming efficiency was defined as the ability to produce colonies in the presence of a drug divided by that ability in the absence of drug.

Assay for Gene Amplification: Genomic DNA was extracted from logarithmically growing cells by the phenol-chloroform extraction method [Blin and Stafford, 1976], and potential gene amplification events were determined by Southern blot analysis as described [Huang et al., 1995a; Choy et al., 1988], using the cDNA fragments as probes noted below. The pCAD142 plasmid containing CAD cDNA, which encodes the CAD protein complex [Shigesada et al., 1985], was used to obtain the 6.3 Kb Hind III fragment as a probe. The pLTR DHFR26 plasmid containing the mouse dihydrofolate reductase gene Chang et al., 1978], provided the 1.3 Kb Bam HI fragment as a probe. The 1487 bp Sal I/Pst I probe for ribonucleotide reductase R2 was prepared from cDNA clone 10 [Huang et al., 1995a; Choy et al., 1988].

Electrophoretic Gel Mobility Shift Assay (EMSA): EMSA was used to determine the presence of wild type p53. Assays were performed essentially as described [Price and Calderwood, 1993], with the following modifications. Cells on 150 mm plates were washed once with ice cold phosphate buffered saline (PBS) and scraped into 1 ml PBS. Cells were pelleted by centrifugation at 1300 g at 4°C for 10 minutes and stored at -80°C. Nuclei were prepared by lysing the pellets in 300 µl buffer A (20 mM HEPES (pH 7.6), 20% glycerol, 10 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA and 0.1% Triton X-100) for 20 minutes on ice. Buffer A also contained 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 mM dithiothreitol (DTT). Nuclei were isolated by centrifugation at 1300 g at 4°C for 10 minutes. Nuclear lysates were prepared by adding 20-40 µl of buffer A containing 500 mM NaCl, 1 mM PMSF and 10 mM DTT to the nuclear pellet and incubating 20 minutes on ice. The extracted nuclei were pelleted by centrifugation at 16,000 g at 4°C; the supernatant was removed and an aliquot was used for protein determination using the Biorad protein assay procedure (Biorad).

The nuclear lysate was incubated with an excess of double stranded p53 consensus binding sequence (GGACATGCCCGGCATGTCC)(SEQ ID No:162) end labeled with [γ -³²P]-ATP using T4 polynucleotide kinase (Boehringer). DNA binding was carried out in buffer containing 20 mM HEPES (pH 7.6), 20% glycerol, 1.5 mM MgCl₂, 0.2 mM EDTA, 1 mM PMSF and 10 mM DTT. Each binding reaction contained 5 µg cell lysate, 10 µg double stranded poly (dI-dC)(Pharmacia), 1.4 ng labeled consensus probe and 100 ng of monoclonal antibody 421 (Santa Cruz) in a total volume of 20 µl. DNA binding was allowed to proceed for 30 minutes at room temperature and the mixture was separated by electrophoresis on 5% nondenaturing polyacrylamide gels. Electrophoresis was carried out at room temperature until the xylene

cyanol tracking dye had run to the bottom of the gel and the free probe had run off the gel.

Statistical Analysis: Analysis of covariance was used to compare dose response data between groups of different cell lines, with the significance level set at $\alpha = 0.05$ [Huang et al., 1995a].

RESULTS

- 5 **Hydroxyurea Resistant Cell Lines with Decreased Sensitivity to Non-selective Drugs.** H-2, H-4, LHF and SC2 are mouse L cell lines selected for resistance to the cytotoxic effects of the antitumor agent, hydroxyurea. These four cell lines exhibited resistance to hydroxyurea in colony forming efficiency experiments, that ranged between approximately 18 (H-2) to 30 (SC2) fold higher than the wild type mouse L cell line from which they were
10 derived [Choy et al., 1998; McClarity et al., 1988]. They also contained elevated levels of ribonucleotide reductase activity that ranged between 2.2 fold (H-2) to 17 fold (LHF and SC2), which was primarily due to increases in the R2 component of ribonucleotide reductase that is limiting for enzyme activity and cell division in proliferating mouse cells. Table 2 shows that the four hydroxyurea resistant cell lines were also less sensitive to the cytotoxic effects of
15 N-(phosphonacetyl)-L-aspartate (PALA) and methotrexate (MTX) in colony forming experiments, when compared to parental wild type mouse L cells. These differences in drug sensitivity are highly significant, with p values of <0.0001 for each of the cell lines when compared to the parental wild type mouse cells.

- Although many mechanisms responsible for drug resistance have been described
20 [Wright, 1989; Kohn, 1996], resistance to MTX and PALA are frequently accompanied by increased levels of the drug targeted gene products, dihydrofolate reductase (DHFR) or CAD (a multifunctional polypeptide containing carbamyl phosphate synthetase, aspartate transcarbamylase and dihydroorotase) respectively, and this often occurs through a mechanism of gene amplification [Huang et al., 1995a; Livingston et al., 1992; Yin et al., 1992;
25 Mai, 1994; Stark, 1993]. Indeed, the principal and perhaps only mechanism for PALA resistance in mouse cells occurs via CAD gene amplification [Stark, 1993]. Therefore, colonies that developed in the presence of normally cytotoxic concentrations of these two drugs were examined for possible gene amplification events. Figure 5 shows that cells that proliferated in the presence of PALA or MTX exhibited increased CAD or DHFR gene copy numbers. In keeping
30 with previous studies [Stark, 1993; Huang et al., 1995b; Otto et al., 1989; Stark et al., 1990], all colonies that developed in PALA and tested (10/10) showed CAD gene amplification. Also as previously reported [Huang et al., 1995b], some but not all colonies that developed in the presence of MTX (3/6) showed DHFR gene amplification.

- Direct Test for a Relationship Between R2 Gene Expression and Decreased Drug**
35 **Sensitivity.** Since hydroxyurea resistant mouse cells contain other biochemical alterations in addition to changes in ribonucleotide reductase [Wright et al., 1989], the relationship between drug sensitivity and increased R2 levels was directly tested with cells containing a retroviral expression vector encoding the mouse R2 sequence, and cells containing the same retroviral

vector but lacking the R2 sequence. B3/mR2 is a population of BALB/c 3T3 cells containing elevated R2 protein due to the presence of a retroviral expression vector encoding R2, and B3/SH is a cell population that has wildtype levels of R2 protein and contains the empty vector as a control. B3/R2c2 is a cloned line with elevated R2 protein selected from the B3/mR2 population.

Consistent with previous reports showing that elevations in R2 gene expression leads to resistance to hydroxyurea, Table 3 shows that B3/mR2 and B3/R2c2 cells are significantly more resistant to the cytotoxic effects of hydroxyurea, at a range of concentrations, when compared to B3/SH cells. These results further demonstrate that B3/mR2 and B3/R2c2 cells express increased levels of an active R2 component of ribonucleotide reductase. B3/mR2 and B3/R2c2 cells were also significantly less sensitive to the cytotoxic effects of PALA and MTX, which act at sites other than ribonucleotide reductase (Table 3). Resistance to these two drugs ranged between approximately 10 fold with 100 nM MTX to more than 100 fold at most concentrations of PALA tested.

Furthermore, Southern blot analysis showed that colonies that developed in the presence of PALA or MTX contained amplifications of CAD or DHFR genes (Figure 6), although as observed with mouse L cells (Figure 5) and as has been reported in other studies [Hurta and Wright, 1992; Hurta et al., 1991], not all colonies that developed in MTX containing medium exhibited DHFR gene amplification. Unlike PALA resistance, MTX resistance in mouse cells can occur through a variety of mechanisms [Otto et al., 1989; Stark et al., 1990; Flintoff, 1989].

The changes in sensitivity to chemotherapeutic compounds exhibited by cells containing elevated levels of the ribonucleotide reductase R2 component were further tested using NIH-3T3 cells containing the R2 expression retroviral vector (Table 4). These cells (N/R2-4) were resistant to hydroxyurea when compared to cells containing the retroviral vector lacking the R2 coding sequence (N/SH). The N/R2-4 cells were also significantly more resistant to MTX. Although the N/R2-4 cells showed a trend towards resistance to PALA when compared to N/SH cells, this trend was not statistically significant. This latter observation indicates that other factors inherent in the genetic differences between the cell lines used in this study, in addition to the increased R2 levels, can influence drug sensitivity responses.

Therefore, the hypothesis that R2 levels are important in determining drug sensitivity characteristics was tested by investigating drug sensitivities after decreasing the levels of R2, through expression of an R2 antisense construct introduced into N/R2-4 cells to produce the N/R2+ASR2 population. Figure 7 shows that the level of R2 protein is markedly reduced in N/R2+ASR2 cells when compared to N/R2-4 cells. The N/R2+ASR2 cells were significantly more sensitive to hydroxyurea, PALA and MTX when compared to N/R2-4 cells (Table 4). Furthermore, sensitivity to these three drugs in the R2 antisense expressing cells was significantly increased when compared to control N/SH cells containing the empty vector

(Table 4).

Mouse 10T $\frac{1}{2}$ cells transfected with activated *ras* and a mutant oncogenic form of p53 are highly resistant to chemotherapeutic agents [Huang et al., 1995b]. The observation that R2 antisense expression can increase sensitivity of NIH-3T3 cells to hydroxyurea, PALA and MTX lead us to test the possibility that cells containing *ras* and mutated p53 may also exhibit reduced drug resistance characteristics in the presence of an R2 antisense sequence. Table 5 shows that this is correct. Cells containing the R2 antisense sequence are significantly more sensitive to hydroxyurea, PALA, and MTX when compared to cells containing the same vector but without R2 in the antisense orientation. These observations suggest that at least one of the determining factors relevant to drug sensitivity of these highly transformed and malignant cells, is ribonucleotide reductase R2 levels.

Evidence that Loss of p53 Protein Function is not Required for R2-Mediated Drug Resistance and Gene Amplification. Inactivation or loss of p53 is a common event associated with the development of tumors and the accompanying decrease in genetic stability observed in malignant cells, including the ability to undergo spontaneous gene amplifications [Liningston et al., 1992; Yin et al., 1992; Takenaka et al., 1995]. Therefore, we tested the possibility that the increased drug resistance properties exhibited by the R2 overproducing B3/mR2 and B3/R2c2 cells may be occurring through a mechanism that results in a loss of wild type p53 activity. It has been demonstrated that p53 is a transcription factor, and that transactivation by wild type p53 but not mutated versions of p53 is sequence-specific, and correlates with its binding to consensus DNA sequences [Takenaka et al., 1995; Kern et al., 1992; Funk et al., 1992]. To determine the presence or absence of wild type p53 function in drug resistant colonies that developed in the presence of PALA, MTX or hydroxyurea, cell extracts were used in electrophoretic gel mobility shift assays (EMSA) [Price and Calderwood, 1993], to test for sequence specific p53 binding activity. Figure 8 shows that drug resistant clones derived from R2 overexpressing cells exhibited wild type p53 binding activity. These observations also agreed with our inability to detect mutant p53 proteins in cells from drug resistant colonies in immunoprecipitation assays using the Pab240 monoclonal antibody [Gannon et al., 1990], which specifically detects common forms of mutant p53.

EXAMPLE 3

ANTISENSE DEOXYRIBONUCLEOTIDE SEQUENCES THAT TARGET RIBONUCLEOTIDE REDUCTASE AND ARE CYTOTOXIC FOR HUMAN TUMOR CELLS.

As shown in the Examples herein above full length antisense constructs of R2 affect the tumorigenicity and/or metastatic competence of tumor cells and susceptibility to chemotherapeutic agents. Applicants therefore investigated the potential of shorter antisense constructs of R1 and R2 for their effect on tumor cells.

MATERIALS AND METHODS

Colony Forming Efficiency and Treatment of Cells with Antisense Constructs: Colony forming

efficiency was determined as previously reported [Huang and Wright, 1994]. The cells were cultured for 24 hours at 37°C in growth medium with 10% fetal bovine serum. The cells were washed in 5ml phosphate buffered saline, pH 7.2, once prior to lipofectin +/- oligonucleotide treatment.

- 5 The oligonucleotides being tested were added to cell cultures in the presence of 2.5 µg of DOTMA/DOPE (Lipofectin; Life Technologies, Inc.) for four hours. The oligonucleotide was tested at 0.2 µM unless otherwise indicated. Controls were the cultures treated with lipofectin but without the oligonucleotide. After 4 hours the medium containing the oligonucleotide was removed and washed with 5 ml of growth medium. The cells were then cultured in growth
10 medium containing 10% fetal bovine serum for seven to ten days. Surviving cells were visualized by methylene blue staining, and colonies were scored. In some experiments cell aliquotes were removed from the culture and viability was determined using the trypan blue exclusion test [Phillips, 1973]. Results were analyzed as percent of surviving cells compared to control cells.

15 RESULTS

- Antisense molecules were identified that target ribonucleotide reductase. As shown below they were cytotoxic for a variety of human tumor cells. Sequences were found that facilitated drug-cytotoxicity for drug resistant tumor cells. That is, at very low non-cytotoxic concentrations, antisense sequences targeting ribonucleotide reductase can sensitize tumor cells
20 to the cytotoxic activity of clinically important chemotherapeutic compounds.

- In initial studies two antisense sequences of 20-mer, designated AS-II-336-20 and AS-II-2229B-20, directed against the R2 mRNA were made and investigated. The first, AS-II-336-20, has the sequence 5'-TCC TGG AAG ATC CTC CTC GC-3' (SEQ ID No:1), and targets the R2 message of human ribonucleotide reductase at nucleotides 336-355, based on the
25 numbering of R2 nucleotides [Pavloff et al., 1992]. The AS-II-2229B-20 sequence is: 5'-TCC CAC ATA TGA GAA AAC TC-3' (SEQ ID No:2), and targets the R2 message at nucleotides 2229-2248. Both AS-II-336-20 and AS-II-2229B-20 were constructed as phosphorothioate sequences to protect against nuclease activity [Anazodo et al., 1995].

- Antisense construct AS-II-336-20 was tested for the ability to inhibit the
30 proliferation of human tumor cells (Hela) in relative colony forming efficiency experiments as described herein above. Hela S3 cells (American Type Culture Collection, Rockville, Maryland, ATCC), and a Hela cell line (Hela 1mM) previously selected for resistance to the antitumor agent, hydroxyurea [Wright et al., 1987], were tested (Table 6). Two experiments were undertaken with Hela S3 cells. With a 4 hour treatment of 0.2 µM antisense construct
35 AS-II-336-20, inhibition of 92% and 82% was seen in colony forming efficiency in two experiments, respectively. The same experiment was repeated with the Hela 1mM cell line and with varying concentrations of the antisense construct AS-II-336-20 (Table 6) with similar results, 0.2 µM was an effective concentration for inhibiting colony formation.

These data show that AS-II-336-20 is a very effective inhibitor of human tumor cell colony forming ability, and it is effective both in inhibiting the proliferation of human tumor cell colony forming ability and in inhibiting the proliferation of human tumor cells that exhibit resistance to another chemotherapeutic compound. Similarly, as shown in Table 6, antisense construct AS-II-336-20 is an effective antitumor compound in experiments performed with the mouse tumor cell line, SC2, which is a highly hydroxyurea resistant mouse L cell line [McClarty et al., 1988].

The antisense sequence AS-II-2229B-20 was also tested for the ability to inhibit the proliferation of human Hela tumor cells in relative colony forming efficiency experiments with results similar to that of AS-II-336-20 as shown in Table 6. These data show that AS-II-2229B-20 is a potent antitumor agent when tested with Hela S3 cells and with the drug resistant Hela 1mM cell line. The antisense construct AS-II-2229B-20 was also tested for the ability to inhibit the proliferation of the human breast cancer cell line MDA435 and found to be very effective (Table 8).

The ribonucleotide reductase R2 antisense construct designated AS-II-2229B-20 was tested for tumor cell cytotoxicity by comparing the results obtained with human tumor and non-tumor cell populations. Hela S3 tumor cells and WI 38 normal non-tumorigenic human cells were used. Tumor cells were found to be much more sensitive to the cytotoxic effects of AS-II-2229B-20 than normal non-tumorigenic cells. For example, analysis of cells three days after antisense exposure indicated that tumor cells were approximately 5-times more sensitive to the cytotoxic effects of AS-II-2229B-20 than normal non-tumorigenic cells averaged over 4-8 determinations.

These results indicate that short oligodeoxyribonucleotide sequences in an antisense orientation are excellent antitumor agents, and suggest that other antisense constructs that target the R2 message may have similar properties. The best antitumor agents would be those that exhibit suitable energy related characteristics important for oligonucleotide duplex formation with their complementary templates, and which show a low potential for self-dimerization or self-complementation [Anazodo et al., 1996]. An analysis of the R2 mRNA using a computer program (OLIGO, Primer Analysis Software, Version 3.4), was carried out to determine antisense sequence melting temperature, free energy properties, and to estimate potential self-dimer formation and self-complementary properties [Anazodo, et al., 1996], of a series of additional antisense sequences (Table 7, SEQ. ID. NOS. 3-102) designed to target the R2 message. Table 7 shows a list of the additional R2 antisense inhibitors, with appropriate properties.

To test the antisense effects of many of these sequences, as phosphorothioate deoxyribonucleotides, they were examined in relative colony forming experiments performed with a series of human tumor cell lines. Many of these antisense constructs, as predicted, are potent inhibitors of human tumor cell proliferation. For results obtained with cancer cells

derived from the bladder, breast, lung, colon, pancreas, prostate, liver and cervix, see Table 12. In addition, *in vivo* studies with AS-II-626-20 were undertaken in C3H/HeN mice as reported in Table 13 and show a significant reduction in metastasis in the antisense treated mice.

Based on Example 2, treatment of human tumor cells with very low concentrations of short antisense sequences was tested to determine if these constructs could sensitize the tumor cells to inhibitory effects of other chemotherapeutic drugs. The concentration used was not cytotoxic in itself as shown in Table 6. The treatment of Hela S3 and Hela 1mM cells with 0.02 μ M of the AS-II-2229B-20 antisense construct increases the sensitivity of these cells to N-(phosphonacetyl)-L-aspartate (PALA) and to methotrexate (MTX) as shown in Table 9. These observations indicate that antisense compounds targeting the R2 message can act synergistically with well known chemotherapeutic agents.

Ribonucleotide reductase is composed of two dissimilar protein components coded by two distinct genes, R1 and R2. Therefore, the results described hereinabove suggest that the R1 message may also be an appropriate target for designing short antisense molecules that have potent antitumor activity. To test this possibility a 20-mer deoxyribonucleotide phosphorothioate sequence in antisense orientation, designated AS-I-1395-20, was constructed and its antitumor abilities were tested. The antisense construct AS-I-1395-20 has the sequence 5'-ACA GGA ATC TTT GTA GAG CA-3' (SEQ ID No:103), and targets the R1 message at nucleotides 1395-1414. As shown in Table 10 it is an effective inhibitor of tumor cell proliferation using Hela S3 cells and Hela 1mM drug resistant cells. These results demonstrate the usefulness of designing antisense sequences that target the R1 message, and suggest that other potential sites may also be effective. Therefore, the R1 mRNA was analyzed in a search for antisense oligodeoxyribonucleotide sequences that exhibit suitable characteristics (as done for R2 mRNA and described above). Table 11 provides a list of additional antisense sequences with characteristics that are consistent with being antitumor agents.

EXAMPLE 4

INHIBITION OF TRANSFORMATION BY R2 ANTISENSE

Utilizing the methods set forth in Examples 1-3, the inhibition of transformation of mammalian cells by treatment with the R2 antisense sequence of the R2 coding region [Fan et al, 1996b] was undertaken. NIH-3T3 mouse cells containing the H-ras oncogene were transfected with either the antisense orientation of the R2 coding sequence or the sense orientation of the R2 coding sequence. The results shown in Fig. 9 demonstrate that in the presence of the R2 antisense construct there was a decrease in transformed foci and reduced soft agar growth (Fig. 9, lane b) compared to the control cells (Fig. 9, lane a). As shown in Example 1, herein above, the R2 coding region can cooperate with H-ras to enhance malignancy as shown by the increased number of transformed foci (Fig. 9, lane c).

Furthermore, colony efficiency assays performed in soft agar as described herein demonstrated similar results. Colony forming efficiencies of 15.6 \pm 6.73 for NIH-3T3 mouse cells

containing the *H-ras* oncogene, 4.4±2.62 for NIH-3T3 mouse cells containing the *H-ras* oncogene and the R2 antisense sequence, and 51±12.29 for NIH-3T3 mouse cells containing the *H-ras* oncogene and the coding region sequence for R2 were seen.

EXAMPLE 5

5 Western blot analysis of AS-II-626-20 inhibition of ribonucleotide reductase R2 protein level in L60 mouse tumor cells. Cells were treated for 4 hours with growth medium supplemented with lipofectin but without antisense oligonucleotides (a) or with lipofectin medium containing 0.2 μM AS-II-626-20 (b). As added controls the tumor cells were also treated for 4 hours with growth medium supplemented with lipofectin and 0.2 μM oligonucleotide scrambled control, 10 which contains the same proportion of nucleotides found in AS-II-626-20 but in a different order (ACGCACCTCAGCTAGTGACAC, SEQ. ID. NO. 164) (c) or with 0.2 μM mismatch oligonucleotide, which contains a four nucleotide mismatch mutation when compared to AS-II-626-20 (TCGC changed to CTGC) (d). Note the significant decrease in R2 protein in tumor cells treated with AS-II-626-20 (b) when compared to the controls (a, c and d).

15 Decrease in R2 protein levels in mouse L60 tumor cells following treatment with a variety of R2 antisense oligonucleotides, as determined by Western blot analysis. Cells were treated for 4 hours with 0.2 μM oligonucleotide in the presence of lipofectin (b to f), or with lipofectin without oligonucleotide as a control (a). (b) Cells treated with AS-II-667-20; (c) cells treated with AS-II-816-20; (d) cells treated with AS-II-1288-20; (e) cells treated with AS-II-1335-20 and, (f) cells treated with AS-II-1338-20. Note the decrease in R2 protein levels in 20 cells treated with antisense oligonucleotides that target the R2 mRNA, in keeping with their abilities to inhibit human tumor cell proliferation (Table 12).

Throughout this application, various publications, including United States patents and published patent applications are referenced by author and year or number. Full citations 25 for the publications are listed below. The disclosures of these publications and patents in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

The invention has been described in an illustrative manner, and it is to be understood that the terminology which has been used is intended to be in the nature of words of 30 description rather than of limitation.

Obviously, many modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.

TABLE 1
INCREASED COLONY FORMATION IN SOFT AGAR BY
***ras*-TRANSFORMED CELLS CONTAINING THE RECOMBINANT R2 VECTOR**

	Cell Line	Colonies (average \pm SE) formed in soft agar with varying cell inoculum ^a		
		10 ³	10 ⁴	10 ⁵
10	C1/SH	0	4 \pm 3	66 \pm 9
	C1/mR2	3 \pm 3	28 \pm 7	347 \pm 45
15	r-2/SH	ND	9 \pm 2	105 \pm 7
	r-2/mR2	ND	24 \pm 1	298 \pm 11
	NR4/SH	0	3 \pm 1	32 \pm 4
	NR4/mR2	2 \pm 1	14 \pm 2	127 \pm 10
	r-3	7 \pm 1	100 \pm 11	ND
20	r-3/mR2	31 \pm 4	309 \pm 17	ND

^a The number of colonies presented were the results obtained in three independent experiments, except those obtained for r-2/SH and r-2/mR2 cells which were the results from single experiments with triplicate dishes. ND, not determined.

TABLE 2

DRUG SENSITIVITIES DETERMINED BY RELATIVE COLONY FORMING EFFICIENCIES
x10⁴

A. PALA

10	DRUG CONC.	CELL LINES				
		W.T.	H2	H4	LHF	SC2
	20 μ M	172.3 \pm 126.3	406.7 \pm 202.2	322.5 \pm 36.4	233.3 \pm 3.6	850.1 \pm 325.2
	30 μ M	50.3 \pm 20.5	39.4 \pm 16.4	84.0 \pm 30.0	78.8 \pm 7.9	187.6 \pm 46.4
15	40 μ M	15.0 \pm 7.0	23.3 \pm 10.4	43.3 \pm 9.6	46.5 \pm 9.9	37.5 \pm 8.7
	50 μ M	3.6 \pm 1.1	7.9 \pm 1.7	23.2 \pm 0.5	25.0 \pm 6.8	47.5 \pm 35.8
	60 μ M	1.3 \pm 0.4	3.6 \pm 0.6	11.1 \pm 1.4	10.7 \pm 3.0	17.6 \pm 1.2

B. MIX

25	CONC.	W.T.	H2	H4	LHF	SC2
	40 nM	11.2 \pm 7.2	52.6 \pm 25.2	44.2 \pm 20.9	143.4 \pm 41.3	880.4 \pm 147.4
	60 nM	12.3 \pm 7.2	73.7 \pm 16.6	34.7 \pm 11.2	63.5 \pm 18.6	566.8 \pm 66.2
30	80 nM	2.2 \pm 1.6	67.7 \pm 20.0	39.3 \pm 18.7	68.2 \pm 19.2	306.6 \pm 61.5
	100 nM	0.8 \pm 0.4	75.3 \pm 10.0	15.1 \pm 8.8	60.8 \pm 16.7	261.8 \pm 39.7
	150 nM	0.5 \pm 0.2	53.3 \pm 9.4	32.3 \pm 13.7	63.9 \pm 16.0	301.6 \pm 76.8

35 The relative colony forming efficiencies are shown \pm se, and the values presented are from 4 to 8 determinations. Statistically significant differences were observed when data obtained with H2 ($p = 0.0004$), H4 ($p \leq 0.0001$), LHF ($p \leq 0.0001$), and SC2 ($p \leq 0.0001$) were each compared to data obtained with the parental wild type (W.T.) cell line.

TABLE 3

DRUG SENSITIVITIES DETERMINED BY
RELATIVE COLONY FORMING EFFICIENCIES $\times 10^{-4}$

A. HYDROXYUREA

10	DRUG CONC	CELL LINES		
		B3/SH	B3/mR2	B3/R2c2
15	0.1 mM	3.3 \pm 1.4	1310 \pm 319.0	830.8 \pm 97.0
	0.4 mM	0.17 \pm 0.19	14.6 \pm 4.0	33.7 \pm 11.0
	0.5 mM	0.21 \pm 0.14	6.5 \pm 4.6	26.9 \pm 11.9
	0.6 mM	0.41 \pm 0.22	5.2 \pm 3.7	12.5 \pm 4.6
	0.8 mM	0.19 \pm 0.62	2.6 \pm 1.4	13.2 \pm 6.4

B. PALA

25	CONC	B3/SH	B3/mR2	B3/R2c2
30	10 μ M	17.9 \pm 11.0	965.0 \pm 529.7	1230.0 \pm 97.0
	20 μ M	0.39 \pm 0.18	120.1 \pm 28.4	55.1 \pm 15.6
	40 μ M	0.35 \pm 0.01	25.0 \pm 4.6	20.2 \pm 6.8
	50 μ M	0.24 \pm 0.14	27.6 \pm 8.9	15.9 \pm 4.0
	60 μ M	0.12 \pm 0.05	25.0 \pm 6.4	18.7 \pm 5.3
	80 μ M	0.17 \pm 0.08	27.1 \pm 6.75	20.0 \pm 4.9

C. MTX

40	CONC	B3/SH	B3/mR2	B3/R2c2
45	20 nM	192.6 \pm 44.6	1055.0 \pm 239.0	382.4 \pm 71.3
	40 nM	15.7 \pm 2.9	62.1 \pm 8.8	60.8 \pm 13.0
	60 nM	6.1 \pm 2.0	76.7 \pm 21.6	64.1 \pm 20.5
	80 nM	2.2 \pm 0.7	17.5 \pm 3.6	20.1 \pm 5.5
	100 nM	1.5 \pm 0.5	12.3 \pm 2.8	21.0 \pm 7.2
	150 nM	3.0 \pm 1.1	23.0 \pm 7.6	33.4 \pm 14.3

The relative colony forming efficiencies are shown \pm SE, and the values presented are from 4 to 12 determinations. Statistically significant differences were observed when data obtained with B3/mR2 or with B3/R2c2 were compared with data obtained with B3/SH (all p values were ≤ 0.0001 for data obtained in the presence of hydroxyurea, PALA or MTX).

TABLE 4

DRUG SENSITIVITIES DETERMINED BY
RELATIVE COLONY FORMING EFFICIENCIES $\times 10^{-4}$

A. HYDROXYUREA

DRUG CONC	CELL LINES		
	N/SH	N/R2-4	N/R2+ASR2
0.3 mM	1.14 ± 0.12	46.1 ± 9.8	0.49 ± 0.34
0.4 mM	0.71 ± 0.17	18.0 ± 6.7	0.14 ± 0.14

B. PALA

CONC	N/SH	N/R2-4	N/R2+ASR2
10 μ M	5.28 ± 1.5	6.22 ± 3.3	1.81 ± 0.8
15 μ M	5.83 ± 2.7	10.0 ± 5.5	0.58 ± 0.3
20 μ M	0.30 ± 0.1	1.71 ± 1.2	0.04 ± 0.04
25 μ M	0.53 ± 0.3	0.8 ± 0.7	0.04 ± 0.04
30 μ M	0.48 ± 0.08	1.03 ± 0.07	0.12 ± 0.12
40 μ M	0.27 ± 0.2	0.14 ± 0.08	0.04 ± 0.04

C. MTX

CONC	N/SH	N/R2-4	N/R2+ASR2
20nM	655 ± 74.8	540 ± 25.1	423 ± 119
40nM	21 ± 12.1	147 ± 4.2	3.5 ± 1.9
60nM	3.4 ± 2.2	62.2 ± 30.7	1.9 ± 1.3
80nM	5.0 ± 5.0	50.4 ± 23.9	2.5 ± 1.5
100nM	4.2 ± 2.5	66.1 ± 32.8	1.1 ± 0.6
150nM	1.4 ± 0.9	21.0 ± 11.5	$0, n=4$

The relative colony forming efficiencies are shown \pm SE, and the values presented are from 4 to 6 determinations. Where 0 is shown the number of determinations using 1×10^5 cells per test is shown as 4 ($n=4$). Statistically significant differences were observed when data obtained with N/SH in the presence of PALA was compared to data obtained with N/R2-4 or with N/R2+ASR2 in the presence of hydroxyurea ($p = 0.0001$ in both cases) or in the presence of MTX ($p = 0.0002$ and 0.032 , respectively). Statistically significant differences were also observed when data obtained with N/SH in the presence of PALA was compared to data obtained with N/R2+ASR2 ($p = 0.002$), but not with data obtained with N/R2-4.

TABLE 5
DRUG SENSITIVITIES DETERMINED BY
RELATIVE COLONY FORMING EFFICIENCIES $\times 10^{-4}$

A. HYDROXYUREA

10	DRUG CONC	CELL LINES			
		RP3/SH	RP3/ASR2	RP6/SH	RP6/ASR2
	0.1 mM	263.6 \pm 19.3	109.8 \pm 43	201.3 \pm 27.2	43.8 \pm 12.3
	0.2 mM	53.6 \pm 13.7	22.9 \pm 3.1	35.5 \pm 8.4	8.6 \pm 2.5
	0.3 mM	20.8 \pm 7.5	6.6 \pm 2.5	12.6 \pm 2.4	4.5 \pm 1.1
15	0.4 mM	5.8 \pm 1.9	1.0 \pm 0.2	10.8 \pm 4.1	1.2 \pm 0.5
	0.5 mM	4.8 \pm 1.9	0.2 \pm 0.1	12.1 \pm 3.9	1.8 \pm 0.9
	0.6 mM	0.7 \pm 0.3	0.3 \pm 0.1	6.6 \pm 2.9	1.5 \pm 0.7
	0.8 mM	0.8 \pm 0.3	0.1 \pm 0.05	1.7 \pm 1.2	0.4 \pm 0.3

B. PALA

	CONC	RP3/SH	RP3/ASR2	RP6/SH	RP6/ASR2
	10 μ M	2569 \pm 338	1183 \pm 384	4619 \pm 648	2083 \pm 960
	20 μ M	123.4 \pm 19.3	86.1 \pm 32.9	1220 \pm 255	368 \pm 154
	30 μ M	45.2 \pm 7.8	19.5 \pm 4.7	450 \pm 129	316 \pm 171
	40 μ M	15.0 \pm 4.9	4.7 \pm 0.6	271 \pm 68	116 \pm 54
30	50 μ M	9.3 \pm 3.6	2.1 \pm 0.8	109 \pm 23	41.7 \pm 23
	60 μ M	3.9 \pm 1.6	0.3 \pm 0.2	55.5 \pm 13	13.2 \pm 6.3

C. MTX

	CONC	RP3/SH	RP3/ASR2	RP6/SH	RP6/ASR2
	20 nM	961.7 \pm 134	485.9 \pm 165	1856 \pm 464	1504 \pm 486
40	40 nM	347.1 \pm 154	77.8 \pm 18	172 \pm 41.3	91.5 \pm 28.1
	60 nM	123.8 \pm 64	18.1 \pm 6.2	77.3 \pm 15.6	49.9 \pm 14.1
	80 nM	66.5 \pm 37	4.4 \pm 0.8	68.7 \pm 16.7	36.0 \pm 6.0
	100 nM	34.8 \pm 21	0.6 \pm 0.06	46.6 \pm 5.6	14.4 \pm 3.8
	150 nM	4.7 \pm 3	0.2 \pm 0.1	11.1 \pm 4.4	3.5 \pm 0.9

The relative colony forming efficiencies are shown \pm SE, and the values presented are from 4 to 10 determinations. Statistically significant differences were observed when data obtained with RP6/SH was compared with data obtained with RP6/ASR2 ($p = 0.0001, 0.0001$ and 0.0001 in the presence of hydroxyurea, PALA and MTX, respectively). Significant differences were also observed when data obtained with RP3/SH was compared with data obtained with RP3/ASR2 ($p = 0.04, 0.0001$ and 0.004 in the presence of hydroxyurea, PALA and MTX, respectively).

TABLE 6

REDUCED COLONY FORMING EFFICIENCY FOLLOWING
TREATMENT WITH R2 ANTISENSE CONSTRUCTS

CELL LINE: HeLa S3

Conc. AS-II-336-20 ^a	% Inhib.	Conc. AS-II-2229B-20 ^b	% Inhib.
0	-	0	-
0.2 μ M	92%	0.05 μ M	50%
0.2 μ M	82%	0.10 μ M	80%
		0.20 μ M	95%
		0.20 μ M	97%

CELL LINE: HeLa 1mM

Conc. AS-II-336-20 ^a	% Inhib.	Conc. AS-II-2229B-20 ^b	% Inhib.
0	-	0	-
0.01 μ M	15%	0.01 μ M	0%
0.05 μ M	25%	0.02 μ M	0%
0.10 μ M	60%	0.03 μ M	21%
0.20 μ M	85%	0.04 μ M	34%
		0.05 μ M	48%
		0.05 μ M	50%
		0.10 μ M	78%
		0.20 μ M	97%
		0.20 μ M	90%

CELL LINE: Mouse SC2

Conc. AS-II-336-20 ^a	% Inhib.
0	-
0.2 μ M	95%

TABLE 7
 ANTISENSE SEQUENCES DESIGNED TO TARGET THE R2 MESSAGE

SEQ ID No:	Name	Sequence 5' - 3'	Tm °C	dG kDa/mol
SEQ ID No:3	AS-II-6-20	ACCCCTCCCATTTGGCTGCGC	62.8	-45.5
SEQ ID No:4	AS-II-13-20	GsCCsTCCGsACCsCTTCsCCsATTsG	60.1	-43.7
SEQ ID No:5	AS-II-14-20	TGCCTCCGACCCCTCCCAATT	60.1	-43.7
SEQ ID No:6	AS-II-16-18	TGCCTCCGACCCCTCCCA	58.4	-40.3
SEQ ID No:7	AS-II-75-20	CsGCGsCGCsTCCsCGGsCCCsTTCsC	72.7	-53.7
SEQ ID No:8	AS-II-75-20	CGCGCGCTCCCGGCCCTTCC	72.7	-53.7
SEQ ID No:9	AS-II-79-14	CGCGCTCCCGGCC	59.1	-38.8
SEQ ID No:10	AS-II-109-20	CsCCCsTCACsTCCsAGCsAGCsCTsT	57.9	-41.8
SEQ ID No:11	AS-II-110-20	ACCCCTCACTCCAGCAGCCT	57.3	-41.2
SEQ ID No:12	AS-II-114-20	GCGGACCCCTCACTCCAGCA	61.8	-43.2
SEQ ID No:13	AS-II-127-12	GCACGGGCGACC	41.7	-28.8
SEQ ID No:14	AS-II-130-20	TGGGACAGGGTGACGGGGCG	67.6	-46.7
SEQ ID No:15	AS-II-134-20	GACGGCTGGGACAGGGTGCA	62.6	-43.2
SEQ ID No:16	AS-II-151-20	GAGCAGCCAGGACAGGACGG	59.3	-41.7
SEQ ID No:17	AS-II-163-20	GsCGsAAGsCAGsAGCsGAGsCAGCsC	62.1	-44.3
SEQ ID No:18	AS-II-166-20	GCAGCGAAGCAGAGCGAGCA	61.4	-43.1
SEQ ID No:19	AS-II-185-20	GGGAGAGCATAGTGGAGGCG	56.0	-40.9
SEQ ID No:20	AS-II-189-20	CGGAGGGAGAGCATAGTGGA	54.1	-39.4
SEQ ID No:21	AS-II-201-20	GCGAGCGGGACACGGAGGGA	63.5	-45.1
SEQ ID No:22	AS-II-217-20	CGGGTCCGTGATGGGCCGGA	69.5	-48.8

SEQ ID No:23	AS-II-225-20	AGCTGCTGCGGGTCCGTGAT	61.4	-43.6
SEQ ID No:24	AS-II-253-14	CCCCCTCAGCGGCG	50.8	-34.4
SEQ ID No:25	AS-II-280-20	CGGCGGCGGTCTCTCTGT	61.8	-44.2
SEQ ID No:26	AS-II-288-12	CGGCGGCGGT	43.2	-29.6
SEQ ID No:27	AS-II-323-20	TCCTCGCGTCTTGCTGGCC	64.1	-45.5
SEQ ID No:28	AS-II-344-20	CCGTGGGCTCCTGGAAGATC	58.0	-41.9
SEQ ID No:29	AS-II-362-20	CTGCTTTAGTTTTCGGCTCC	51.2	-39.2
SEQ ID No:30	AS-II-391-17	CGGCTCATCCTCCACGC	54.5	-37.3
SEQ ID No:31	AS-II-404-20	GGTTTTCTCTCAGCAGCGGC	56.4	-41.4
SEQ ID No:32	AS-II-412-20	GCGGCGGGGGTTTTCTCTCA	62.8	-45.8
SEQ ID No:33	AS-II-414-20	AAGCGGCGGGGTTTTCTCT	60.7	-45.8
SEQ ID No:34	AS-II-425-20	GGAAGATGACAAAGCGGCGG	59.1	-43.0
SEQ ID No:35	AS-II-439-20	ATGGTACTCGATGGGAAGA	50.8	-37.8
SEQ ID No:36	AS-II-472-20	AGCCTCTGCCTTCTTATACA	46.1	-35.8
SEQ ID No:37	AS-II-494-20	CCTCCTCGGCGTCCAAAAG	60.4	-44.3
SEQ ID No:38	AS-II-496-16	TCCTCGGCGGTCCAAA	54.8	-37.0
SEQ ID No:39	AS-II-549-20	TATCTCTCCTCGGGTTTCAG	48.4	-36.7
SEQ ID No:40	AS-II-579-20	GCAAAGAAAGCCAGAACATG	50.0	-37.2
SEQ ID No:41	AS-II-619-20	TCGCTCCACCAAGTTTCAT	52.1	-38.3
SEQ ID No:42	AS-II-626-20	GGCTAAATCGCTCCACCAAG	53.9	-40.3
SEQ ID No:43	AS-II-634-20	AACTTCTTGGCTAAATCGCT	48.0	-37.6
SEQ ID No:44	AS-II-667-20	GAAGCCATAGAAACACGGG	53.9	-40.3
SEQ ID No:45	AS-II-784-20	GACACAAGGCATCGTTCAA	50.9	-36.8
SEQ ID No:46	AS-II-798-20	TCTGCCTTCTTGTGACACA	48.0	-34.9

SEQ ID No:47	AS-II-816-20	ATCCAGCGCAAGGCCAGTC	60.9	-43.7
SEQ ID No:48	AS-II-861-20	GCAAAGGCTACAACACGTTC	50.0	-37.1
SEQ ID No:49	AS-II-890-20	AACCGGAAAAGAAAATGCCT	52.2	-40.4
SEQ ID No:50	AS-II-909-20	CAGAATATCGACGCAAAAGA	48.2	-36.5
SEQ ID No:51	AS-II-933-20	GGCATCAGTCCTCGTTTCTT	50.8	-37.7
SEQ ID No:52	AS-II-981-20	TGTAAACCTCATCTCTGCT	46.2	-35.0
SEQ ID No:53	AS-II-1001-20	TCAGGCAAGCAAAATCAG	51.3	-37.2
SEQ ID No:54	AS-II-1006-20	GAACATCAGGCAAGCAAAAT	49.4	-37.1
SEQ ID No:55	AS-II-1023-20	TTGTGTACCAAGGTGTIGAA	45.9	-33.9
SEQ ID No:56	AS-II-1040-20	CTCTCTCTCCGATGGTTTG	51.1	-37.7
SEQ ID No:57	AS-II-1048-20	TTCTCTTACTCTCTCTCCG	45.2	-35.0
SEQ ID No:58	AS-II-1144-20	GTATTGCTTCATTAGAGTGC	41.6	-33.0
SEQ ID No:59	AS-II-1182-20	CCCAGTTCAGCATAAGTCT	48.4	-36.5
SEQ ID No:60	AS-II-1197-20	AAAACCTTGCTAAACCCAG	48.3	-37.8
SEQ ID No:61	AS-II-1217-20	CAAATGGGTCTCTACTCTG	43.7	-33.8
SEQ ID No:62	AS-II-1224-20	ATAAAGTCAAATGGGTTCTC	42.6	-34.0
SEQ ID No:63	AS-II-1254-20	TTAGTCTTTCCTTCCAGTGA	43.8	-33.9
SEQ ID No:64	AS-II-1278-20	TCGCCTACTCTCTTCTCAA	46.8	-35.6
SEQ ID No:65	AS-II-1288-20	CCTCTGATACTCGCCTACTC	45.6	-35.1
SEQ ID No:66	AS-II-1302-20	GACATCACTCCCATCTCTG	48.7	-35.3
SEQ ID No:67	AS-II-1335-20	GCATCCAAGGTAAAAGAATT	45.6	-36.1
SEQ ID No:68	AS-II-1338-20	TCAGCATCCAAGGTAAAAGA	47.4	-35.9
SEQ ID No:69	AS-II-1342-20	GAAGTCAGCATCCAAGGTAA	46.7	-35.3
SEQ ID No:70	AS-II-1345-20	TTAGAAGTCAGCATCCAAGG	47.0	-35.6

SEQ ID No:71	AS-II-1362-20	GCACATCTTCAGTTCATTA	42.4	-32.8
SEQ ID No:72	AS-II-1364-20	GGGCACATCTTCAGTTCATT	48.9	-36.2
SEQ ID No:73	AS-II-1381-20	AAAAATCAGCCAAGTAAGGG	48.1	-38.0
SEQ ID No:74	AS-II-1390-20	ATGGAAAAAAAAAATCAGCC	48.1	-38.0
SEQ ID No:75	AS-II-1438-20	TTCATGGTGTGGCTAGTTGG	50.8	-36.8
SEQ ID No:76	AS-II-1499-20	AGGACTGGTTGTGAGGTAGC	48.1	-35.7
SEQ ID No:77	AS-II-1517-20	CCAGCACTATAACAGACAG	42.2	-32.8
SEQ ID No:78	AS-II-1538-20	TTCTGGCAAAAGGTGATACT	46.5	-35.6
SEQ ID No:79	AS-II-1560-20	GTAAGTCACAGCCAGCCAGG	52.2	-37.8
SEQ ID No:80	AS-II-1581-20	ACTGCCATTGTCACTGCTAT	47.0	-34.9
SEQ ID No:81	AS-II-1659-20	TGGCTGTGCTGGTTAAAGGA	53.2	-38.7
SEQ ID No:82	AS-II-1666-20	TTTTAACTGGCTGTGCTGGT	50.0	-37.2
SEQ ID No:83	AS-II-1700-20	ATTAAATCTGCGTTGAAGC	46.8	-36.6
SEQ ID No:84	AS-II-1768-20	TATCGCCGCCGTGAGTACAA	56.5	-40.9
SEQ ID No:85	AS-II-1773-20	GCTATTATCGCCGCCGTGAG	57.1	-42.6
SEQ ID No:86	AS-II-1775-12	ATCGCCGCCGTG	42.9	-29.5
SEQ ID No:87	AS-II-1790-20	GAAACCAAATAATCAAGCT	43.4	-34.9
SEQ ID No:88	AS-II-1819-20	TTAGTGGTCAGGAGAATGTA	41.7	-32.5
SEQ ID No:89	AS-II-1976-20	TGGCACCAACTGACTAATAT	44.5	-34.2
SEQ ID No:90	AS-II-1989-20	CCTGTCTTCTATCTGGCACC	48.6	-36.2
SEQ ID No:91	AS-II-2009-20	GCCACAGGATAAAACACAA	47.7	-35.9
SEQ ID No:92	AS-II-2026-20	CCCAGGACACTACACAAGCC	51.8	-37.5
SEQ ID No:93	AS-II-2044-20	TCAGAGGGGGCAGAGAATCC	55.4	-40.2
SEQ ID No:94	AS-II-2067-20	TCCTTTATCCACAACACTC	46.3	-35.0

SEQ ID No:95	AS-II-2083-20	CCTTGCCCTGAGAGATTCCT	52.3	-39.0
SEQ ID No:96	AS-II-2083-20	CsCTsTGsCCsCTsGAsGAsGAsTTsCCsT	52.3	-39.0
SEQ ID No:97	AS-II-2128-20	GGCCCAGATCACCCCTAAAT	54.3	-40.9
SEQ ID No:98	AS-II-2151-20	AAACGGCTTCTCACACATAT	46.3	-35.4
SEQ ID No:99	AS-II-2164-20	GAGAAATAAAATGAAACGGC	46.2	-36.6
SEQ ID No:100	AS-II-2182-20	CGTTGAGGAAAATACAGTGA	45.1	-34.3
SEQ ID No:101	AS-II-2229A-20	GCTCCACATATGAAAACCTC	46.1	-35.2
SEQ ID No:102	AS-II-2372-20	CACACAACCTACTTACACCA	42.7	-32.3

Footnotes for Table 7

Name includes the following

AS = antisense;

II = R2

The first number indicates the first nucleotide position in the R2 mRNA sequence.

The second number indicates the length of the sequence segment.

The sequence AS-II-2229A shown in the Table and the sequence AS-II-2229B described in the text are alternate sequences, with 2229A chosen from the version of R2 in GENBANK (submitted by Pavloff) and 2229B chosen from the version published by Pavloff et al., J. DNA Sequencing and Mapping, 2:227-234, 1992.

Sequences were fully thioated unless partial thioation is indicated (s).

¹TM°C = Melting temperature of oligonucleotide duplex formed.

²dG = Free energy values for oligonucleotide-complement dimer formation.

In addition to the above analysis, estimates of potential dimer formation (D), potential self-complementary interactions (H), and the potential to bind to sequences in the R2 message other than the target sequence (B) were obtained. Analysis and estimates described above were obtained by using the computer modelling program OLIGO Primer Analysis Software, Version 3.4 (distributed by National Biosciences). The program allows the determination of Tm°C and dG values, and also provides a qualitative estimation of the D, H and B parameters indicating "no potential", "some potential" or essentially "complete potential". In choosing the oligonucleotide sequences we gave high priority to sequences that exhibited high Tm°C and dG values, which are important for tight binding of antisense molecules to their complementary strands, and high priority to antisense sequences that had estimates of no potential in D, H and B. Of the three categories (D,H,B) the most important ones were D and H, since B (i.e. binding to other regions of the R2 mRNA in addition to the precise target sequence) may enhance rather than compromise oligonucleotide activity. Most of the sequences shown in Table 7 had no potential in the D and H categories. Some sequences exhibited "some potential" in D or H and were later found in tumor cell growth inhibition studies to be effective (Table 12) and therefore were also included in Table 7. We found that this approach to choosing antisense oligonucleotide inhibitors was extremely effective, since the vast majority of the chosen sequences exhibited anti-tumor properties as shown in Table 12.

TABLE 8
TREATMENT WITH AN R2 ANTISENSE CONSTRUCTS

CONSTRUCT	CONC. (μ m)	COLONY FORMING INHIBITION OF: MDA435
AS-II-2229B-20	0.02	25%
	0.03	56%
	0.05	78%
	0.10	94%
	0.20	99%

TABLE 9

SYNERGISTIC EFFECT OF AS-II-2229B-20 ANTISENSE CONSTRUCT

Cells	Drug	Drug Conc.	AS-II-2229B-20 ^a 0.02_M	Relative Colony Forming Efficiency ^c
Hela S3	PALA ^a	20μM	-	350 ± 50
	PALA	20μM	+	90 ± 10
Hela S3	MTX ^a	40μM	-	118 ± 32
	MTX	60μM	-	116 ± 13
	MTX	40μM	+	25 ± 5
	MTX	60μM	+	0
Hela 1mM	PALA	20μM	-	377 ± 21
	PALA	30μM	-	311 ± 9.5
	PALA	20μM	+	108 ± 7.5
	PALA	30μM	+	101 ± 2.0
Hela 1mM	MTX	40μM	-	28 ± 10
	MTX	60μM	-	12 ± 0.5
	MTX	40μM	+	6.5 ± 5.5
	MTX	60μM	+	3.5 ± 0.5

^a PALA = N-(phosphonacetyl)-L-aspartate^a MTX = methotrexate^b - = no treatment^b + = treatment provided^c The values are the average of two experiments.

TABLE 10

REDUCED COLONY FORMING EFFICIENCY FOLLOWING
TREATMENT WITH R1 ANTISENSE CONSTRUCT

CELL LINE: HeLa S3

<u>Conc. AS-I-1395-20a</u>	<u>% Inhib.</u>
0	-
0.2 μ M	75% (Exp. 1)
0.2 μ M	77% (Exp. 2)

CELL LINE: HeLa 1mM

<u>Conc. AS-I-1395-20a</u>	<u>% Inhib.</u>
0	-
0.01 μ M	0
0.05 μ M	30%
0.10 μ M	60%

CELL LINE: Mouse SC2

<u>Conc. AS-I-1395-20a</u>	<u>% Inhib.</u>
0	-
0.2 μ M	76%

TABLE 11
 ANTISENSE SEQUENCES DESIGNED TO TARGET THE R1 MESSAGE

SEQ ID No:	Name	Sequence 5' - 3'	Tm °C	dG kDa/mol
SEQ ID No:104	AS-I-35-20	GTT CCA GCC AGA CAG CAC TT	51.7	-37.3
SEQ ID No:105	AS-I-37-20	GAG TTC CAG CCA GAC AGC AC	52.0	-37.0
SEQ ID No:106	AS-I-85-20	CAG AGT GGG AAG GGT TAG GT	49.7	-37.5
SEQ ID No:107	AS-I-91-20	AGG TGA CAG AGT GGG AAG GG	52.7	-38.2
SEQ ID No:108	AS-I-129-20	GAC TGG ACT GCG GCT CTA AA	52.1	-38.3
SEQ ID No:109	AS-I-203-20	ATG ACT CGT TCT TGG CGG CC	58.6	-42.4
SEQ ID No:110	AS-I-239-20	CAA AGC TTC TGG ATT CGA GA	49.6	-37.1
SEQ ID No:111	AS-I-287-20	TTC ATG GTG ATC TGA GCA GG	50.6	-36.2
SEQ ID No:112	AS-I-300-20	GCC TTG GAT TAC TTT CAT GG	48.9	-37.3
SEQ ID No:113	AS-I-348-20	TTC AGC AGC CAA AGT ATC TA	45.4	-34.9
SEQ ID No:114	AS-I-395-20	GCC AGG ATA GCA TAG TCA GG	48.9	-36.9
SEQ ID No:115	AS-I-439-20	CIT TCT TTG TTT CIT TGT GC	44.5	-34.6
SEQ ID No:116	AS-I-504-20	GGG AGA GTG TTT GCC ATT AT	48.2	-36.7
SEQ ID No:117	AS-I-520-20	TTG ACT TGG CCA CCA TGG GA	58.2	-40.8
SEQ ID No:118	AS-I-540-20	GGC CAG AAC AAT ATC CAA TG	49.5	-37.2
SEQ ID No:119	AS-I-556-20	TCA GGC GAT CIT TAT TGG CC	54.2	-40.5
SEQ ID No:120	AS-I-635-20	TTC AAC AAA TAA GAC CGC TC	47.2	-36.1
SEQ ID No:121	AS-I-658-20	TTT CAG CCA CIT TTC CAT TG	50.3	-37.5
SEQ ID No:122	AS-I-662-20	GGT CIT TCA GCC ACT TTT CC	50.4	-37.9
SEQ ID No:123	AS-I-782-20	TTG AAG AGA GTG GGC GAA GC	54.4	-39.6
SEQ ID No:124	AS-I-786-20	AGC ATT GAA GAG AGT GGG CG	54.3	-39.5

SEQ ID No:125	AS-I-809-20	GAA AGT TGC GGG CGG TTG GT	60.6	-44.3
SEQ ID No:126	AS-I-843-20	GCT GTC ATC TTT CAT ACT CA	41.9	-32.2
SEQ ID No:127	AS-I-908-20	CCA ATT CCT CCA GCA GAC TT	50.8	-37.8
SEQ ID No:128	AS-I-923-20	CAA CTC ACA GCA ACA CCA AT	48.1	-34.8
SEQ ID No:129	AS-I-932-20	GCC CGA ATA CAA CTC ACA GC	52.2	-38.2
SEQ ID No:130	AS-I-967-20	AAT TGC CAT TAG TCC CAG CA	52.2	-38.8
SEQ ID No:131	AS-I-1051-20	ATG CCC CAG GAC GCT TGT TC	58.5	-42.2
SEQ ID No:132	AS-I-1074-20	CCA AGG CTC CAG GTA AAT AG	48.4	-37.6
SEQ ID No:133	AS-I-1134-20	ACG CTG CTC TTC CTT TCC TG	53.7	-39.6
SEQ ID No:134	AS-I-1162-20	TCC AAA GAG CAA AGA AAA GA	47.0	-36.1
SEQ ID No:135	AS-I-1258-20	CCT CTC CCC AAA CCT CAT CC	54.7	-40.2
SEQ ID No:136	AS-I-1311-20	AAC TTT GCG GAC ACG ACC TT	53.7	-39.5
SEQ ID No:137	AS-I-1370-20	GGG GTG CCT GTT TCC GTC TG	58.9	-42.0
SEQ ID No:138	AS-I-1418-20	TTC TGC TGG TTG CTC TTT CG	53.1	-38.7
SEQ ID No:139	AS-I-1421-20	AGG TTC TGC TGG TTG CTC TT	50.6	-37.6
SEQ ID No:140	AS-I-1513-20	GGG CCA GGG AAG CCA AAT TA	57.6	-43.4
SEQ ID No:141	AS-I-1662-20	GGG GCG ATG GCG TTT ATT TG	58.8	-44.0
SEQ ID No:142	AS-I-1666-20	CAA TGG GGC GAT GGC GTT TA	60.1	-44.0
SEQ ID No:143	AS-I-1785-20	TTC CAG AGC ACC ATA ATA AA	45.1	-35.1
SEQ ID No:144	AS-I-1818-20	TGG GCC CTG CTC CTT GGC AA	64.3	-45.7
SEQ ID No:145	AS-I-1970-20	GGC ATC GGG GCA ATA AGT AA	54.1	-41.0
SEQ ID No:146	AS-I-1976-20	GCT GTA GGC ATC GGG GCA AT	58.5	-42.9
SEQ ID No:147	AS-I-2119-20	CAT GCC ATA GGC CCC GCT CG	64.0	-46.4
SEQ ID No:148	AS-I-2198-20	AGT TGC TTC AGG TCA TCA GG	49.0	-36.0

SEQ ID No:149	AS-I-2251-20	CAG CTG CCA TCT TGA GAA CA	51.1	-36.6
SEQ ID No:150	AS-I-2304-20	CTC AGC AAT GTG GAT GTT CA	48.9	-35.0
SEQ ID No:151	AS-I-2364-20	AGT CTT CAA ACC CTG CTT CC	50.0	-37.6
SEQ ID No:152	AS-I-2370-20	CAT CCC AGT CTT CAA ACC CT	50.4	-37.5
SEQ ID No:153	AS-I-2414-20	GTG AAC TGG ATT GGA TTACG	46.1	-35.2
SEQ ID No:154	AS-I-2491-20	TGG CTG CTG TGT TCC TCT CC	55.0	-38.8
SEQ ID No:155	AS-I-2556-20	CTT CCA AGT CTT TCC TCA GG	48.0	-36.4
SEQ ID No:156	AS-I-2629-20	TAC CAC CTC AAG CAA ACC CA	52.9	-38.4
SEQ ID No:157	AS-I-2650-20	CAA CAG GGT CCA GCA AAG CC	56.8	-40.9
SEQ ID No:158	AS-I-2769-20	TCC GTT TTT TTT TTC TTT TT	46.2	-37.5
SEQ ID No:159	AS-I-2863-20	TGC TAA ATG GGT GAT GAA AC	47.5	-35.8
SEQ ID No:160	AS-I-2922-20	CCC ACC AGT CAA AGC AGT AA	50.2	-36.9
SEQ ID No:161	AS-I-2594-20	CTC AAG AAG TAG TTT GGC TA-3'	41.6	-33.2

Footnotes for Table 11

Name includes the following:

AS = antisense

I = R1

The first number indicates the first nucleotide position in the R1 mRNA sequence.

The second number indicates the length of the sequence segment.

$^1T_m^{\circ}C$ = Melting temperature of oligonucleotide duplex formed.

2dG = Free energy value for oligonucleotide-complement dimer formation.

In addition to the above analysis, estimates of potential dimer formation (D), potential self-complementary interactions (H), and the potential to bind to sequences in the R1 message other than the target sequence (B), were obtained. Analyses were performed as described in the Footnote to Table 7, and criteria used to select the sequences shown in Table 11 were as indicated in the Footnote to Table 7.

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TABLE 12Table 12: Reduced Relative Colony Forming Efficiency of Human Tumor Cells Following Treatment with 0.2 μ M of Various Antisense Oligodeoxynucleotide Phosphorothionates Targeting the R2 Message, Expressed As % Inhibition

Name (Re)	T24	HCT116	A549	MDA-MB-231	MIA PaCa-2	PC-3	HepG2	Hela S3	T-47D	H596	Colo320
AS-II-6-20	73.85	ND	ND	88.4	95.15	89.21	97.89	ND	ND	ND	ND
AS-II-13-20*	18.99	6.95	32.3	45.45	ND	52.38	24.11	19.85	15.33	19.68	ND
AS-II-14-20	77.59	ND	ND	91.24	47.93	92.76	88.4	ND	ND	ND	ND
AS-II-16-18	25.74	78.57	81.1	62.59	ND	89.48	75.89	68.7	7.13	34.5	ND
AS-II-75-20*	73.42	44.4	60.08	49.3	97.38	68.25	35.4	93.01	32.95	ND	ND
AS-II-75-20	95.83	ND	ND	95.14	52.07	83.46	97.89	ND	ND	ND	ND
AS-II-79-14	38.4	45.56	79.17	48.6	38.89	85.32	70.81	28.64	70.81	ND	ND
AS-II-109-20*	24.89	6.76	15.14	22.38	54.24	61.51	18.08	46.83	20.63	7.28	ND
AS-II-110-20	87.78	71.69	89.38	90.92	47.51	92.06	97.14	53.98	ND	ND	ND
AS-II-114-20	87.45	86.1	83.51	76.22	90.05	92.66	78.72	79.25	90.83	46.3	ND
AS-II-127-12	50.63	54.34	69.33	38.46	53.24	79.56	71.75	86.45	37.54	ND	ND
AS-II-130-20	51.94	57.98	86.48	ND	82.11	74.66	94.28	ND	ND	ND	ND
AS-II-134-20	ND	ND	ND	ND	ND	77.51	ND	ND	ND	ND	ND
AS-II-151-20	ND	78.09	84.28	41.64	75.38	85.68	89.58	66.75	95.89	69.12	90.12
AS-II-163-20*	5.49	29.05	37.13	22.73	9.88	7.14	18.64	45.8	9.81	32.08	ND
AS-II-166-20	68.99	73.84	81.1	29.02	91.36	74.11	78.72	80.1	91.4	61.99	ND

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TABLE 12 (continued)

Name (Re)	T24	HCT116	A549	MDA- MB-231	MIA PaCa-2	PC-3	HepG2	Hela S3	T-47D	H596	Colo320
AS-II-185-20	21.94	ND	71.51	17.4	29.32	4.37	53.44	19.38	94.52	24.53	ND
AS-II-189-20	18.57	86.78	76.57	39.86	70.52	73.12	57.86	76.67	96.63	26.15	ND
AS-II-201-20	96.2	45.56	90.55	25.17	70.22	65.08	59.32	90.87	98.53	49.6	ND
AS-II-217-20	65.02	61.85	ND	52.7	87.38	87.41	99.55	ND	ND	ND	ND
AS-II-225-20	73.23	59.5	92.9	ND	95.44	80.06	ND	96.99	ND	ND	ND
AS-II-253-14	19.41	53.28	61.62	45.37	ND	67.26	42	65.18	27.09	0.81	ND
AS-II-280-20	90.56	69.42	61.81	79.14	53.94	77.51	97.14	41.79	ND	ND	ND
AS-II-288-12	30.38	67.57	70.49	52.1	30.09	74.01	65.89	57.63	ND	12.67	ND
AS-II-323-20	ND	55.8	91.24	ND	97.55	79.76	96.39	ND	ND	ND	ND
AS-II-344-20	ND	ND	ND	ND	ND	80.06	ND	ND	ND	ND	ND
AS-II-362-20	89.63	62.81	61.81	85.83	34.2	75.78	95.78	45.69	ND	ND	ND
AS-II-391-17	ND	ND	ND	ND	26.35	93.25	60.64	ND	ND	ND	ND
AS-II-404-20	84.26	ND	52.17	85.83	17.84	77.08	84.79	58.37	ND	ND	ND
AS-II-412-20	22.2	27.98	43.78	ND	55.25	73.96	26.23	ND	ND	ND	ND
AS-II-414-20	11.67	19.1	12.44	ND	36.11	60.94	30.89	ND	ND	ND	ND
AS-II-425-20	90.37	ND	57.38	89.75	65.2	75.65	97.89	63.09	ND	ND	ND
AS-II-435-20	67.84	64.7	76.46	ND	92.69	77.66	73.04	ND	ND	ND	ND
AS-II-472-20	69.26	67.23	96.99	ND	97.13	90.7	ND	ND	ND	ND	ND

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TABLE 12 (continued)

Name (Re)	T24	HCT116	A549	MDA-MB-231	MIA-PaCa-2	PC-3	HepG2	Hela S3	T-47D	H596	Colo320
AS-II-494-20	54.23	50.28	33.85	54.78	25.31	80.6	93.37	48.62	ND	ND	ND
AS-II-496-16	78.48	70.85	74.45	45.8	ND	88.84	54.8	52.21	10.79	ND	ND
AS-II-549-20	45.46	47.83	30.57	40.13	17.01	ND	84.04	27.8	ND	ND	ND
AS-II-579-20	76.68	69.08	95.49	66.89	97.55	88.16	94.28	ND	ND	ND	ND
AS-II-619-20	86.3	ND	65.67	91.08	39.83	88.01	92.02	31.22	ND	ND	ND
AS-II-626-20	76.79	70.46	95.14	90.21	75.62	83.23	75.89	67.92	66.12	ND	ND
AS-II-634-20	83.52	ND	57.76	92.44	ND	77.86	95.78	48.94	ND	ND	ND
AS-II-667-20	70.48	76.9	70.3	ND	85.26	91.8	88.23	ND	ND	ND	ND
AS-II-784-20	87.23	78.09	83.8	33.92	62.04	88.99	80.89	81.48	85.39	ND	ND
AS-II-798-20	84.72	64.46	70.49	83.92	34.65	83.21	89.46	56.42	ND	ND	ND
AS-II-816-20	73.91	88.22	78.4	ND	93.21	94.08	93.08	ND	ND	ND	ND
AS-II-861-20	73.5	74.2	95.78	89.98	97.3	87.33	96.08	ND	ND	ND	ND
AS-II-890-20	82.07	ND	81.6	88.2	66.02	87.93	ND	ND	ND	ND	ND
AS-II-909-20	78.57	ND	78.68	45.96	46.13	84.86	ND	ND	ND	ND	ND
AS-II-933-20	64.84	67.24	53.52	64.89	35.68	86.91	79.97	26.86	ND	ND	ND
AS-II-981-20	86.3	66.84	74.25	91.48	ND	85.16	95.03	69.43	ND	ND	ND
AS-II-1001-20	86.11	55.58	71.36	82.17	64.21	85.94	90.36	ND	ND	ND	ND
AS-II-1006-20	61.49	45.56	61.62	ND	47.93	92.58	89.31	41.79	ND	ND	ND

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TABLE 12 (continued)

Name (Re)	T24	HCT116	A549	MDA- MB-231	MIA PaCa-2	PC-3	HepG2	Hela S3	T-47D	H596	Colo320
AS-II-1023-20	58.26	ND	34.52	ND	42.82	87.63	ND	ND	ND	ND	ND
AS-II-1040-20	59.49	70.08	85.82	ND	43.52	40.08	77.78	71.87	64.76	ND	ND
AS-II-1048-20	40.32	42.63	65.67	66.88	33.4	84.38	77.56	39.19	ND	ND	ND
AS-II-1144-20	63.9	54.25	61.81	ND	46.89	80.21	92.17	50.57	ND	ND	ND
AS-II-1182-20	94.51	88.13	80.06	ND	84.72	92.76	92.23	90.61	92.41	ND	ND
AS-II-1197-20	90.3	84.85	89.15	50.35	70.68	74.4	76.32	82.68	81.95	ND	ND
AS-II-1217-20	66.36	68.68	91.49	ND	34.85	81.03	ND	ND	ND	ND	ND
AS-II-1224-20	38.31	41.78	55.06	ND	17.22	80.66	76.05	14.8	ND	ND	ND
AS-II-1254-20	41.53	28.54	36.74	ND	3.32	73.31	83.28	7.64	ND	ND	ND
AS-II-1278-20	65.42	ND	ND	90.68	57.05	85.31	ND	ND	ND	ND	ND
AS-II-1288-20	56.75	66.43	61.04	ND	80.71	93.55	80.41	ND	ND	ND	ND
AS-II-1302-20	70.56	71.98	93.17	92.2	23.86	79.01	ND	ND	ND	ND	ND
AS-II-1335-20	59.95	67.87	78.59	ND	78.78	90.04	72.98	ND	ND	ND	ND
AS-II-1338-20	63.16	74.73	63.93	ND	79.17	93.75	80.41	ND	ND	ND	ND
AS-II-1342-20	59.76	73.74	65.67	ND	73.77	89.84	82.2	ND	ND	ND	ND
AS-II-1345-20	51.26	65.7	73.1	94.11	77.39	89.58	75.42	ND	ND	ND	ND
AS-II-1362-20	ND	78.47	83.9	70.22	44.14	77.38	80.41	ND	ND	ND	ND

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TABLE 12 (continued)

Name (Re)	T24	HCT116	A549	MDA-MB-231	MIA PaCa-2	PC-3	HepG2	Hela S3	T-47D	H596	Colo320
AS-IL-1364-20	66.59	77.29	95.59	93.87	59.34	79.01	ND	ND	ND	ND	ND
AS-IL-1381-20	71.37	89.48	86.02	44.41	73.77	75	62.34	80.53	93.62	45.28	ND
AS-IL-1390-20	61.13	62.18	88.31	66.89	82.77	76.76	90.21	ND	ND	ND	ND
AS-IL-1438-20	43.7	ND	51.27	69.06	42.13	83.96	ND	ND	ND	ND	ND
AS-IL-1499-20	82.81	83.01	87.8	41.26	81.17	77.28	77.5	87.56	96.67	78.3	ND
AS-IL-1517-20	ND	ND	ND	ND	ND	91.75	ND	ND	ND	ND	ND
AS-IL-1538-20	67.29	51.28	90.34	ND	50.62	84.71	96.84	ND	ND	ND	ND
AS-IL-1560-20	32.49	85.81	84.19	46.15	83.8	78.37	73.63	82.16	86.6	71.16	ND
AS-IL-1581-20	68.22	66.85	90.55	ND	24.07	85.83	93.07	ND	ND	ND	ND
AS-IL-1659-20	74.09	ND	54.7	42.86	42.54	81.56	ND	ND	ND	ND	ND
AS-IL-1666-20	71.71	ND	54.82	26.71	49.72	86.06	ND	ND	ND	ND	ND
AS-IL-1700-20	70.94	ND	77.28	30.75	34.52	90.63	ND	ND	ND	ND	ND
AS-IL-1768-20	74.56	ND	86.8	91.56	60.36	86.36	ND	ND	ND	ND	ND
AS-IL-1773-20	15.19	75.58	70.11	44.76	45.68	70.04	58.19	80.27	84.38	66.04	ND
AS-IL-1775-12	85.54	54.44	63.55	48.6	27.78	78.17	43.97	68.61	ND	18.6	ND
AS-IL-1790-20	ND	ND	ND	ND	ND	87.86	ND	ND	ND	ND	ND
AS-IL-1819-20	53.74	ND	ND	90.68	20.02	85.46	83.89	ND	ND	ND	ND

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TABLE 12 (continued)

Name (Re)	T24	HCT116	A549	MDA- MB-231	MIA PaCa-2	PC-3	HepG2	Hela S3	T-47D	H596	Colo320
AS-II-1976-20	ND	ND	ND	89.6	ND	88.16	ND	ND	ND	ND	ND
AS-II-1989-20	77.43	78.47	83.9	54.9	70.22	77.38	80.7	61.41	90.83	56.33	ND
AS-II-2009-20	61.84	69.92	93.32	96.25	93.74	83.36	96.99	ND	ND	ND	ND
AS-II-2026-20	95.46	81.47	88.81	77.1	87.65	95.29	94.54	83.79	93.41	84.16	ND
AS-II-2044-20	53.63	49.34	25.55	19.11	24.48	74.48	62.35	24.55	ND	ND	ND
AS-II-2067-20	49.6	47.16	64.71	49.68	41.08	85.94	90.36	24.88	ND	ND	ND
AS-II-2083-20	82.43	87.46	90.65	68.88	71	ND	93.64	84.58	89.32	82.98	87.28
AS-II-2083-20*	9.52	41.16	31.73	ND	ND	82.03	46.14	6.96	48.61	49.87	52.54
AS-II-2128-20	83.74	ND	87.31	91.3	39.23	88.89	ND	ND	ND	ND	ND
AS-II-2151-20	79.83	ND	79.19	95.14	62.15	84.86	ND	ND	ND	ND	ND
AS-II-2164-20	61.84	50.08	91.15	69.03	89.36	83.36	93.07	ND	ND	ND	ND
AS-II-2182-20	67.76	77.66	90.97	84.95	56.43	85.91	95.48	ND	ND	ND	ND
AS-II-2229A-20	50.34	93.01	69.72	ND	33.61	89.58	73.26	63.15	58.82	ND	ND
AS-II-2372-20	61.13	64.7	96.41	90.09	94.36	86.06	ND	ND	ND	ND	ND

Legend to Table 12

- The antisense oligonucleotides were fully thioated unless indicated (*), as described in Table 7.
- The values for relative colony-forming efficiencies are averages obtained from 2-8 determinations.
- ND = not determined.
- The various cell lines were obtained from the American Type Culture Collection, Rockville, Maryland.

Information about these human cancer cells:

T24	=	bladder cell carcinoma
HCT116	=	colon cell carcinoma
A549	=	lung cell carcinoma
MDA-MB-231	=	breast cell adenocarcinoma
MIA PaCa-2	=	pancreatic cell carcinoma
PC-3	=	prostate cell adenocarcinoma
HepG2	=	hepatocellular carcinoma
HelaS3	=	cells isolated from a carcinoma of the cervix
T-47D	=	breast ductal carcinoma
H596	=	lung adenosquamous carcinoma cells
Colo320	=	colon cell adenocarcinoma

TABLE 13

Metastatic Characteristics of r-3 Mouse 10T^{1/2} Tumor Cells in
Syngeneic Mice Following Treatment with the Antisense
Oligonucleotides, AS-II-626-20

Oligonucleotide * Treatment	Frequency of Mice with Tumors	Number of Lung Tumors (mean \pm SE)
none	4/4	6.0 \pm 1.58
0.2 μ M	1/4	0.25 \pm 0.25

* 10⁵ cells either treated for 4 hours with lipofectin without oligonucleotide supplement (none) or with lipofectin containing 0.2 μ M AS-II-626-20, were injected intravenously (tail vein) into C3H/HeN syngeneic mice and lung tumors were analyzed as previously described (Damen, J.E., Greenberg, A.H. and Wright, J.A. *Biochim. Biophys. Acta.*, 1097:103-110, 1991). The r-3 cell line is highly malignant and has been described previously (Taylor, W.R., Egan, S.E., Mowat, M., Greenberg, A.H. and Wright, J.A. *Oncogene*, 7:1383-1390, 1992). The differences observed between the AS-II-626-20 treated and untreated groups were statistically significant (p value = 0.027). Clearly, AS-II-626-20 treated tumor cells exhibited a marked reduction in metastatic potential.

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WE CLAIM:

1. An antisense oligonucleotide having a sequence which is complimentary to a nucleic acid sequence from a ribonucleotide reductase gene and comprises at least seven
5 nucleotides or nucleotide analogues.
2. An oligonucleotide according to claim 1 which is complimentary to an mRNA region from a ribonucleotide reductase gene.
- 10 3. An oligonucleotide according to claim 2 which is complimentary to an mRNA region from the ribonucleotide reductase R2 gene.
4. An oligonucleotide according to claim 3 wherein the oligonucleotide has a nucleic acid sequence shown in one of SEQ. ID NOS 1 to 102 as shown in Table 7 or an analogue
15 thereof.
5. An oligonucleotide according to claim 3 wherein the oligonucleotide has a nucleic acid sequence shown in SEQ. ID NOS 1, 2, 12, 16, 18, 21, 25, 29, 34, 42, 44, 45, 46, 52, 53, 59, 60, 64, 65, 66, 68, 69, 70, 72, 73, 74, 76, 78, 79, 80, 90, 91, 92, 96, 99, 100 or 102 as shown in Table 7.
6. An oligonucleotide according to claim 3 wherein the oligonucleotide has a nucleic acid sequence shown in Table 12.
7. An oligonucleotide according to claim 2 which is complimentary to a region
25 from the ribonucleotide reductase R1 gene.
8. An oligonucleotide according to claim 7 wherein the oligonucleotide has a nucleic acid sequence shown in one of SEQ. ID NOS 103-161 as shown in Table 11, or an analogue thereof.
9. An oligonucleotide according to claim 7 wherein the oligonucleotide has a nucleic acid sequence shown in SEQ. ID NO 103 as shown in Table 11, or an analogue thereof.
10. A pharmaceutical composition for modulating tumor cell growth comprising
35 at least one antisense oligonucleotide according to any one of claims 1 to 9, in admixture with a physiologically acceptable carrier or diluent.
11. A pharmaceutical composition for inhibiting tumor cell proliferation

comprising at least one antisense oligonucleotide according to any one of claims 1 to 9 in admixture with a physiologically acceptable carrier or diluent.

12. A pharmaceutical composition for increasing the sensitivity of a tumor cell to a chemotherapeutic drug comprising at least one antisense oligonucleotide according to any one of claims 1 to 9 in admixture with a physiologically acceptable carrier or diluent.

13. A pharmaceutical composition for modulating the growth of a tumor cell that is resistant to a chemotherapeutic drug comprising at least one antisense oligonucleotide according to any one of claims 1 to 9 in admixture with a physiologically acceptable carrier or diluent.

14. A use of an antisense oligonucleotide according to any one of claims 1 to 9 to prepare a medicament for modulating tumor cell growth.

15. A use of an antisense oligonucleotide according to any one claims 1 to 9 to prepare a medicament for inhibiting tumor cell proliferation.

16. A DNA sequence comprising a transcriptional initiation region and a sequence encoding an antisense oligonucleotide according to any one of claims 1 to 9.

17. A vector comprising a DNA sequence according to claim 16.

18. An antisense oligonucleotide according to any one of claims 1 to 9 wherein the oligonucleotide exhibits reduced dimer formation and reduced self-complementary interactions.

19. A method of increasing sensitivity of neoplastic cells to chemotherapeutic drugs in a mammal by contacting the tumor with at least one antisense oligonucleotide according to claim 1 to 9 and a chemotherapeutic drug.

20. The method as set forth in claim 19 wherein the chemotherapeutic drug is selected from hydroxyurea, MTX and PALA.

21. A method according to claim 19 wherein the antisense oligonucleotide and chemotherapeutic drug are administered at a noncytotoxic dose.

22. A method according to claim 19 wherein the antisense oligonucleotide and

chemotherapeutic drug are administered at a cytotoxic dose.

23. A method according to claim 19 wherein the antisense oligonucleotide is administered at a noncytotoxic dose and the drug is administered at a cytotoxic dose.

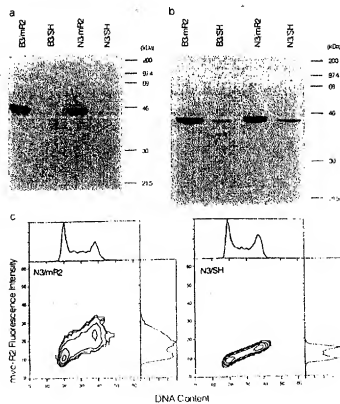
24. A method according to claim 19 wherein the antisense oligonucleotide is administered at a cytotoxic dose and the drug is administered at a noncytotoxic dose.

25. A method for modulating tumor cell proliferation comprising contacting a
10 cell with an effective amount of at least one antisense oligonucleotide according to claim 1 to 9.

26. A method of evaluating if a compound inhibits transcription or translation of a ribonucleotide reductase gene and thereby affects cell proliferation comprising transfecting a cell with an expression vector comprising a recombinant molecule comprising a nucleic acid
15 sequence encoding ribonucleotide reductase, and the necessary elements for the transcription or translation of the nucleic acid; administering a test compound; and comparing the level of expression of the ribonucleotide reductase with the level obtained with a control in the absence of the test compound.

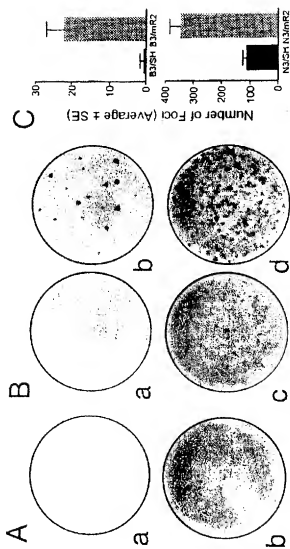
20 27. A method of evaluating a compound for its ability to regulate a Ras signalling pathway by assaying for an agonist or antagonist of the interaction of R2 and Raf-1 and/or Rac-1 comprising providing a reaction mixture containing R2 and Raf-1 and/or Rac-1 under conditions which permit the interaction of R2 and Raf-1 and/or Rac-1, in the presence of a test compound; detecting the formation of complexes between R2 and Raf-1 and/or Rac-1 or
25 activation of a Ras signalling pathway; and comparing to a control reaction in the absence of the test substance, wherein lower levels of complexes or activation in the reaction mixture indicate that the test compound interferes with the interaction of R2 and Raf-1 and/or Rac-1, and higher levels indicate that the test compound enhances the interaction of R2 and Raf-1 and/or Rac-1.

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FIGURE 1

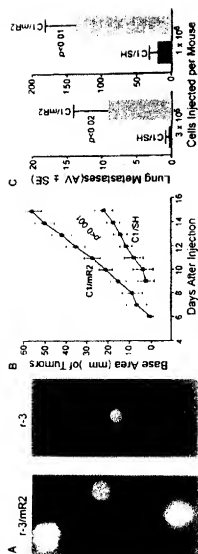
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FIGURE 2



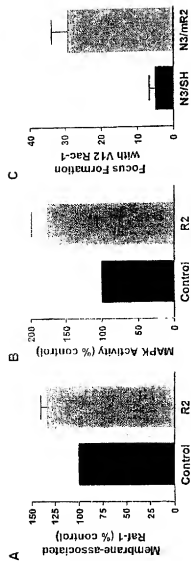
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FIGURE 3

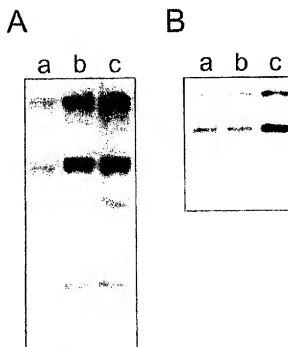


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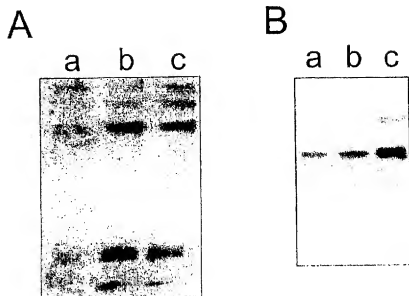
FIGURE 4



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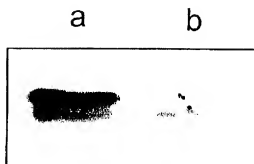
FIGURE 5

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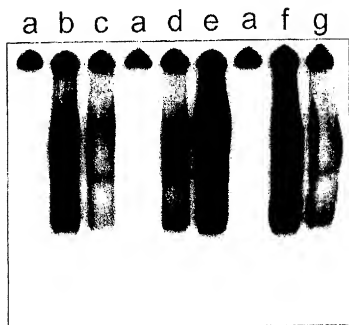
FIGURE 6

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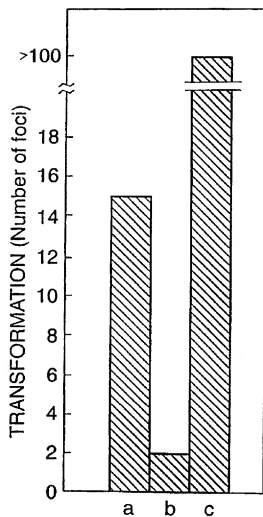
FIGURE 7



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FIGURE 8

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FIGURE 9

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FIGURE 10